Identification of Cell Type Enriched Cofactors of NF-κB Dependent Transcription

Dissertation

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Summary

A multi-cellular organism has many different types of cell and each is characterised by a particular morphology and metabolism to perform a specialised function. Variation occurs between the cell types despite the fact that every cell in an organsism has the same genetic material. The physiology of each individual cell type is due to differences in the expression of proteins, the molecular workers of the cellular factory. Proteins are coded for by genes and one of the major levels of regulation of gene expression is through transcription. Transcription of genes is the molecular process where RNA polymerase II (RNAPII) transfers the DNA code into an RNA code. This process is regulated at multiple steps by transcription factors and their cofactors. The chromatin structure also has a role in transcription regulation since the DNA must be accessible to transcriptional regulators for RNAPII activity.

Nuclear Factor kappa B (NF-κB) is a family of inducible transcription factors responsible for transcription of a wide variety of genes upon cellular stimulation with a huge array of factors. RelA (also called p65) is a major member of this family and is expressed in all cell types. The transcription of NF-κB regulated genes may be regulated according to the cell type due to a cell type specific chromatin status and cell type enriched cofactors. The aim of this thesis was to identify novel cell type enriched cofactors of RelA(p65) dependent transcription. A proteomics approach was used to isolate and identify proteins that interact with the transcription activation domain (TAD) of RelA(p65). Known and novel cofactors of RelA(p65)-TAD were identified. Among others, a novel RelA(p65)-TAD interaction partner, called MYB binding protein 1a (MYBBP1a), was found as a cell type enriched factor. MYBBP1a was detected to repress NF-κB regulated transcription *in vivo* and *in vitro* and to compete with a co-activator for interaction with the TAD of RelA(p65). The work presented in this thesis shows the identification of cell type enriched cofactors of NF-κB and indicates that the cofactor occupancy of RelA(p65) could be important in the regulation of cell type specific gene expression.

Zusammenfassung

Ein vielzelliger Organismus besteht aus vielen verschiedenen Zelltypen, die aufgrund verschiedener Morphologien und Metabolismen spezialisierte Funktionen übernehmen können. Obwohl jede Zelle in einem Organismus das gleiche genetische Material aufweist, treten Unterschiede zwischen den verschiedenen Zelltypen auf. Die Physiologie jedes Zelltyps beruht auf der unterschiedlichen Expression von Proteinen, den molekularen Regulatoren der zellulären Maschinerie. Proteine wiederum werden von Genen kodiert; diese werden zum grossen Teil auf Ebene der Transkription reguliert. Gentranskription ist der molekulare Prozess, bei welchem die RNS Polymerase II (RNAPII) die Information der DNS in RNS überschreibt. Dieser Prozess wird auf verschiedenen Ebenen durch Transkriptionsfaktoren und ihre Kofaktoren reguliert. Auch die Chromatinstruktur spielt eine Rolle, da die DNS für die Regulatoren der RNAPII-Aktivität zugänglich sein muss.

Nukleärer Faktor kappa B (NF-κB) ist eine Familie von induzierbaren Transkriptionsfaktoren. Nach zellulärer Stimulation durch eine Vielzahl von Faktoren sind sie verantwortlich für die Transkription einer breiten Anzahl von Genen. Das wichtigste Mitglied dieser Familie ist RelA (auch p65 genannt), welches in allen Zelltypen exprimiert wird. Für die Transkription von Genen, welche von NF-κB reguliert werden, sind auch der zelltyp-spezifische Chromatinstatus und Kofaktoren, welche in diesem Zelltyp angereichert sind, wichtig. Das Ziel dieser Doktorarbeit war es, neue zelltyp-spezifische Kofaktoren der RelA(p65)- abhängigen Transkription zu identifizieren. Dafür wurde eine spezielle Proteomics-Methode angewendet um Proteine zu isolieren und identifizieren, welche mit der Transkriptions-Aktivierungs-Domäne (TAD) von p65 interagieren. So wurden bekannte und auch neue Kofaktoren von p65-TAD identifiziert. Einer dieser neuen Interaktionspartner, MYB Bindendes Protein 1a (MYBBP1a), stellte sich als zelltyp-spezifisch angereicherter Faktor heraus. MYBBP1a reprimierte NF-κB regulierte Transkription *in vivo* und *in vitro* und konkurrierte mit einem bekannten Ko-Aktivator um die Bindung an die TAD von p65. Die in dieser Doktorarbeit präsentierten Ergebnisse bestätigen die Identifikation von zelltyp-spezifischen Kofaktoren für NF-κB und weisen darauf hin, dass die Komplexbildung von RelA(p65) mit Kofaktoren eine wichtige Rolle in der Regulation der Genexpression spielt.

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Abbreviations

AhR	Aromatic hydrocarbon receptor
ACF	ATP-utilizing chromatin assembly and remodelling factor
AML1	Acute myeloid leukaemia protein 1
ATM	Ataxia telangiectasia mutated
ATP	Adenosine 5'-triphosphate
ATR	ATM and Rad3 related protein
BAF	Brg or Brm associated factors
BRG	Brahma-related gene 1
BRM	Brahma
BUR	Base unpairing region
CBP	cAMP-response element binding (CREB) binding protein
ChIP	Chromatin immunoprecipitation
CHRAC	Chromatin-accessibility complex
CIITA	Class II transactivator
CoA	Coenzyme A
co-REST	Co-repressor of RE1 silencing transcription factor
CT	Carboxyl-terminal
CTD	Carboxyl terminal domain
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA dependent protein kinase, catalytic subunit
ER	Estrogen receptor
GTF	General transcription factor
H	Histone
HAT	Histone acetyl transferase
HBO1	HAT bound to ORC1
HDAC	Histone de-acetylase
HIV1-LTR	Human immunodeficiency virus 1 long terminal repeat
IFN	Interferon
Ig	Immunoglobulin
IKK	IκB kinase
IL	Interleukin
IR	Ionising radiation
IRF	Interferon regulator factor
kbp	Kilo base pairs
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MAR	Matrix attachment region
MORF	MOZ (monocytic leukemia zinc finger) -related factor
mRNA	Messenger ribonucleic acid
MYBBP1a	Myb binding protein 1a
N-CoA-1,2,3	Nuclear receptor co-activator 1,2,3
N-CoR	Nuclear receptor co-repressor
NELF	Negative elongation factor
NES	Nuclear export signal

NF-κB	Nuclear Factor kappa B
NHEJ	Non-homologous end joining
NHR	Nuclear hormone receptor
NLS	Nuclear localisation signal
NoRC	Nucleolar remodeling complex
NRD	Negative regulatory domain
NT	Amino-terminal
NuRD	Nucleosome remodeling and deacetylation
OPT	Oct-1/PTF/transcription
ORF	Open reading frames
PARP1	Poly ADP-ribosylpolymerase 1
PCAF	P300/CBP associated factor
PGC-1α	PPARγ co-activator 1α
PIC	Pre-initiation complex
PKA	Protein kinase A
PMA	Phorbal 12-myristate 13-acetate
PML	Promyelocytic leukemia
PPARγ	Peroxisome proliferation activated receptor γ
PRMT	Protein arginine methyltransferase
P-TEFb	Positive transcription elongation factor b
RHD	Rel Homology Domain
RNA	Ribonucleic acid
RNAPII	RNA Polymerase II
RSF	Remodeling and spacing factor
SAGA	Spt-Ada-Gcn5-acetyltransferase
SAP	Sin associated protein
SATB1	Special AT-rich binding protein 1
SET	Su(var), E(z) and trithorax
SIR	Silence information regulator
SMRT	Silencing mediator for retinoic acid and thyroid hormone receptor
SRC-1,2,3	Steroid receptor co-activator 1, 2, 3
SREBP1a	Sterol regulatory element binding protein 1a
SSU72	Suppressor of *sua*7 protein 72
STAGA	Spt3-TAF31-GCN5L acetylase
STAT2	Signal transducer and activator of transcription 2
TAD	Transcription activation domain
TBP	TATA box binding protein
TF	Transcription factor
TFII	Transcription factor of RNAPII
TFIIIC	Transcription factor of polIII C
TFTC	TBP free TAF complex
tip60	HIV Tat-interacting protein 60 kDa
TNFα	Tumour necrosis factor alpha
TRRAP	Transactivating/transformation domain associated protein
TSA	Trichostatin A
WCRF	Williams syndrome transcription factor related
WICH	WSTF-ISWI chromatin remodeling complex

Introduction

1 The mechanism of RNA Polymerase II Transcription

Within the cell the transcription of particular genes is regulated by external and internal molecular signals (reviewed in [1]). External signals such as cytokines, hormones and stress signals, together with internal signals such as nutrient and Adenosine 5'-triphosphate (ATP) levels activate many different signal transduction pathways. These pathways lead to the activation of appropriate cellular responses, which is often dependent on the regulation of gene expression through modulation of transcription. Both the intensity and duration of transcription of a particular gene can be regulated. The intensity of transcription can be regulated by variation of the number of polymerases transcribing at once. Since transcription of genes often occurs in waves the duration of transcription can be increased by increasing the number of transcription waves or by elongating each wave.

RNA polymerase II (RNAPII) transcription involves a cycle of events (reviewed in [2]). It starts with the building of a large protein complex, containing the factors required for transcription initiation (pre-initiation complex formation) and finishes with termination of RNA synthesis and the recycling of the protein factors involved, ready for the next round of transcription (transcription termination and re-initiation).

Regulation of transcription is tightly linked to regulation of chromatin structure (reviewed in [3]). In order for transcription to occur the transcription machinery must have access to the DNA substrate. This often requires adjustment of the chromatin structure at gene regulatory elements. After transcription some of the chromatin adjustments are retained to maintain the potential of the gene to be actively transcribed once again. Transcription factors (TFs) and transcription cofactors are the proteins that regulate transcription both positively and negatively by altering the accessibility of the DNA substrate as well as influencing protein complex formation during the different steps of transcription.

1.1 The General Transcription Initiation Machinery

1.1.1 RNA Polymerase II

In eukaryotic cells there are three RNA polymerases, RNA polymerase I, II and III. RNAPII is the polymerase that synthesises the protein coding messenger RNA (mRNA) in the cell. The structure of the 12 subunit elongating RNAPII complexed with DNA substrate has been shown at least three times at high resolution [4-6]. The structures give insight into the mode of action of RNAPII and the model for catalysis. DNA enters a groove in the enzyme and is unwound by the effect of positive and negative charged residues of RNAPII pushing and pulling about 12-14 nucleotides of the template strand away from the non-template strand of DNA. The rNTPs are positioned in a pocket of the enzyme, directly opposite the template strand at position +1 (the transcription start site). RNA and DNA hybrids are formed and survive for around 7 nucleotides. The RNA/DNA hybrids are unravelled, the DNA strands re-dimerise, and the nascent RNA and duplex DNA leave the enzyme at right angles to each other (reviewed in [7] and [8]). The largest subunit of RNAPII is Rpb1, and it has a carboxyl terminal domain (CTD) essential for the regulation of transcription as well as other RNA processing events. The CTD contains phosphorylatable repeats of YSPTSPS (52 in humans and 26 in yeast) and seems to be unstructured [9].

1.1.2 The transcription cycle

Transcription by RNAPII occurs in cycles, consisting of pre-initiation complex (PIC) assembly, open complex formation, catalysis of the first diester bond, promoter escape, elongation, termination and re-initiation. The control of the transcription cycle is tightly linked with the phosphorylation and dephosphorylation of the serines and threonines in the CTD of RNAPII. The recruitment of various factors involved in transcription and mRNA processing is dependent on the phosphorylation state of the CTD (Figure 1) (reviewed in [2]). In the first step of the transcription cycle, PIC formation, the CTD of RNAPII is not phosphorylated [10, 11].

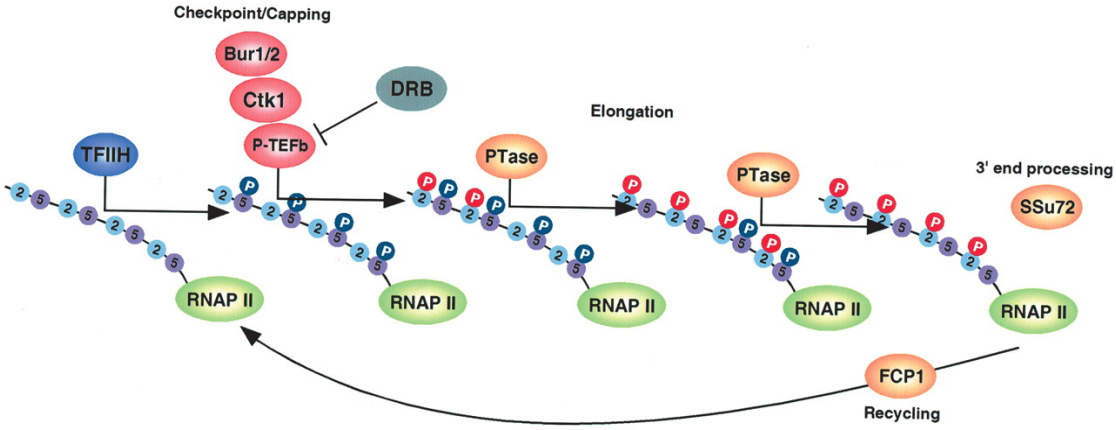

Figure 1. The transcription cycle and CTD phosphorylation events. RNAPII CTD is phosphorylated at serine 5 by TFIIH CAK upon transcription initiation. The transcription elongating complex is stalled to promote mRNA capping and is released by Bur1/2, Ctk1 (yeast) and P-TEFb (human). Upon elongation RNAPII CTD is phosphorylated at serine 2 by P-TEFb. Dephosphorylation by phosphatases (PTase), such as SSU72 and FCP1, occurs at termination/re-initiation allowing recycling of RNAPII and 3' processing of the nascent RNA. Picture taken from [12].

PIC formation requires several general transcription factors (GTFs), which are assembled at the promoter in order. RNAPII, transcription factor of RNAPII F and B (TFIIF and TFIIB) and TATA box binding protein (TBP) are the minimal protein components required to accurately initiate transcription *in vitro* from various supercoiled promoters [13-15]. Transcription from non-supercoiled DNA *in vitro* requires further GTFs including TFIIE and TFIIH [13-15]. Mediator is a large protein complex, often associated with RNAPII to form a holoenzyme and is also important for transcription of many genes. Chromatin immunoprecipitation (ChIPs) show that *in vivo* all the GTFs and Mediator are recruited to many different promoters but not open reading frames (ORFs) in yeast implying that the proteins are generally required for transcription initiation [16]. The structure of a RNAPII, Mediator, TBP, TFIIB and TFIIF complex has been modelled by comparison of electron micrographs, crystal structures and structural predictions of the components [8]. However, no Med-RNAPII-TFIIF complexes were found in *S. cerevisiae* although 20% of cellular RNAPII is associated with Mediator and 50% of RNAPII is associated with TFIIF, implying that these factors may not interact with RNAPII at once [17]. For PIC formation at TATA box containing promoters the order of GTF recruitment is TBP (or TFIID), TFIIB plus TFIIA, TFIIF plus RNAPII, TFIIE plus TFIIH (reviewed in [18]). In some cases it has been shown that RNAPII joins the PIC as a holoenzyme with Mediator, while in other cases Mediator precedes or follows RNAPII recruitment to the promoter.

During open complex formation the DNA strands are separated by RNAPII as described in Section 1.1.1. This is an ATP dependent reaction and is enhanced by TFIIE and TFIIH [19]. Transcription is initiated by formation of the first phosphodiester bond, and phosphorylation at serine 5 of the CTD by the Cdk activating kinase (CAK) subdomain of the multi-protein TFIIH. Promoter escape/clearance refers to the stage of transcription between initiation and elongation. Small RNAs are formed and the RNAPII has a tendency to spontaneously dissociate from the DNA. RNAPII instability is reduced when the transcript is longer than 9 bp, and is completely stable when the transcript is longer than 23 nucleotides [20]. RNAPII CTD is phosphorylated at serine 5 during promoter clearance and during stalling. DRB (5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole) sensitivity inducing factor (DSIF) and negative elongation factor (NELF) interact with the phosphoserine and recruit the mRNA capping machinery (Figure 1).

Transcription elongation is the process whereby full-length mRNA transcripts are produced. The elongating RNAPII is present in a large multi-protein complex, the RNAPII transcription elongation complex (TEC). TEC is distinct from the PIC as GTFs (except TFIIF) have dissociated from RNAPII and remained at the promoter. RNAPII must overcome transcriptional pausing, transcription arrest and transcription termination. RNAPII may naturally pause during transcription due to physical barriers present such as histones and mRNA processing factors. Many protein factors affect the pausing and also the rate of elongation e.g. TFIIF, TFIIS, FCP-1 (TFIIF associating RNAPII CTD phosphatase), P-TEFb (positive transcription elongation factor b), elongins, TREX (transcription/export complex), proteosome components, and ELLs (eleven nineteen lysine rich leukaemia gene proteins) (reviewed in [2, 12]). Other elongation factors are involved in the displacement of repressive chromatin proteins. The structure of the elongating RNAPII indicates how the long transcript cleavage factor, TFIIS, could stretch from the outside of RNAPII to the inside, to approach the active site with a hairpin like structure to stimulate the nuclease activity of RNAPII [6]. In this way stalled RNAPII would be released from the RNA substrate to re-initiate transcription. The structure of the elongating RNAPII complex also indicates that once the transcript is more than 18 nucleotides long it is accessible to the solvent and therefore mRNA processing enzymes [6]. During transcriptional elongation RNAPII CTD is heavily phosphorylated at serine 2 by various kinases

such as P-TEFb (CyclinT and Cdk9) [21]. Co-transcriptional splicing factors and elongation factors are recruited to the RNAPII (Figure 1).

Transcription initiation, elongation and termination are tightly coordinated as some of the same proteins are involved in the different steps. In *S. cerevisiae* the promoter and terminator DNA sequences have been shown to loop together [22]. The mechanism of termination of transcription and the protein factors involved are still not well characterised (reviewed in [23, 24]). The DNA regions involved consist of a pause site or termination region as well as a polyA tract. RNAPII transcribes past the polyA tract, into the "terminator region". However, the rapid degradation of the RNA means that all the nascent mRNA isolated is only as long as the polyA tract. Upon transcription termination mRNA is cleaved, poly-adenylated and transported to the cytoplasm [23, 24].

Transcription re-initiation involves the recycling of some GTFs to the promoter and dephosphorylation of RNAPII CTD by phosphatases (reviewed in [2]). Serine 5 is dephosphorylated by SSU72 and small CTD phosphatases (SCPs) while serine 2 is dephosphorylated by FCP1. TFIIF was also shown to be important for re-initiation *in vitro* [17].

1.2 The Substrate for Transcription

The substrate for transcription is tremendously complex. A wide variety of regulatory DNA sequence elements bind the transcription machinery. The transcription of a gene is effected by the DNA regulatory elements present, the local environment of the gene with respect to nucleosome positioning and histone modifications, the higher order structure of the chromatin that packages the gene and the position of the gene in the nucleus (reviewed in [25], [3] and [26]). In general, actively transcribed genes are thought of as being more accessible, at the levels of higher order chromatin structure and individual nucleosomes.

1.2.1 The core promoter

The core promoter is defined as the minimal DNA sequence that can direct accurate transcription initiation by the RNAPII machinery. The core promoter differs slightly from gene to gene. In general it is around 70 bp long spanning the initiation site (position +1) and contains a selection of motifs (reviewed in [18]). These are TATA

(TBP binding element), Inr (initiator), BRE (TFIIB recognition element) and DPE (downstream core promoter element) (Figure 2).

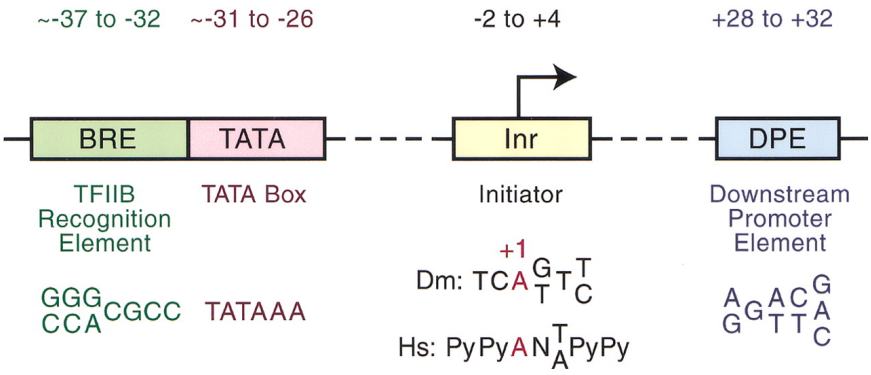

Figure 2. The core promoter. The motifs involved in transcription from RNAPII promoters are shown. Transcription starts from +1 in TATA containing promoters. The Initiator in *Homo sapiens* (Hs) and *Drosophila melanogaster* (Dm) has the consensus sequences shown where Py is pyrimidine, T (thymine) or C (cytosine) and N is any base. Where 2 or more bases are shown at the same position, either can be present. Picture taken from [18].

TBP and TRFs (TBP related factors) bind directly to the TATA box. TATA is not present in all promoters, and current estimates based on computer predictions are that it is present in approximately 20-25% of all gene regulatory regions [27]. Transcription normally initiates at A+1 of the Inr consensus sequence (Py-Py-A+1-N-T/A-Py-Py). RNAPII and TFIID via TAF1 ($TAF_{II}250$) and TAF2 ($TAF_{II}150$) can interact with Inr [28, 29]. Two sequence specific DNA binding proteins, TFII-I and YingYang, also bind Inr at selected promoters [30, 31].

The DPE binds TFIID (but not TBP) via TAF6 ($dmTAF_{II}60$) and TAF9 ($dmTAF_{II}40$) and is present more commonly in TATA less promoters [32]. It is located at +28 to +32 and the distance between Inr and DPE is critical. Negative cofactor 2 can repress TATA dependent transcription whilst activating DPE dependent (TATA less) transcription, indicating the mechanism of transcription initiation by these two DNA elements is different [33].

BRE has been shown to act positively or negatively on transcription and to bind to TFIIB [34]. CpG islands are stretches of DNA (about 0.5 to 2 kbp) that contain promoters (often TATA/DPE less), and GC boxes (binds the Sp1 transcription factor). In general, transcription from these promoters starts from multiple weakly active sites, unlike the specific single start site in TATA/DPE containing promoters.

The function of the core promoter is to correctly position the RNAPII machinery as well as to regulate transcription initiation via regulation of GTFs. Some promoters contain two TATA boxes that can be differentially used according to the cellular state, also resulting in regulation of transcription. Different TATA boxes are responsive to different stimuli, presumably because of differences in TFIID compositions and TAF activities (reviewed in [18]).

1.2.2 Enhancers, proximal promoters, silencers and Insulators

Proximal promoters are situated around -250 to +250 bp from the transcription start site, while enhancers and silencers can be close to the start site or many kbp up or downstream from the start site [35]. A diagram of a typical simple yeast control region of a gene and a complex metazoan control region of a gene is shown in Figure 3. Promoters and enhancers generally activate transcription (together with trans-activating regulators), whereas silencers repress transcription. Metazoan enhancers often regulate tissue or cell type specific transcription, are around 500 bp long, and have an average of about ten binding sites for many different transcription factors (TFs). Enhancers integrate signals, to regulate transcription of a specific gene according to the cell type and cellular status (reviewed in [36]).

Insulators prevent inappropriate regulation of promoters by enhancers when positioned between the two elements. They also reduce the effect of chromosome position on transcription of synthetically integrated genes (reviewed in [37]). Insulator elements and the protein complexes associated with them are thought to have a role in regulation of the higher order chromatin structure (Section 1.2.3).

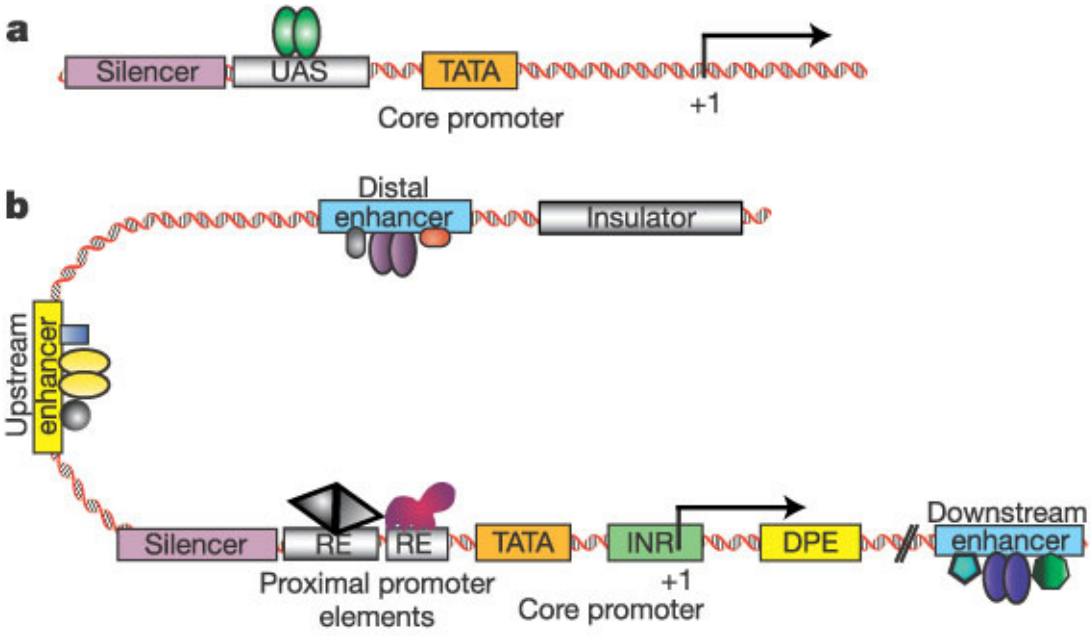

Figure 3. Control elements for a typical yeast gene (a) and a more complex metazoan gene (b). TATA, INR, DPE, Insulator, Silencers and Enhancers are described in the text. Enhancers can be downstream, upstream or distal from the gene and bind to transcription factors (ovals and rectangles). Transcription factors also bind to response elements (RE) in the proximal promoter and upstream activating sequences (UAS) in yeast. Picture taken from [38].

1.2.3 Nucleosome structure

In cells the DNA sequence elements described above are coiled around an octamer of histones to form nucleosomes. The core particle of the nucleosome consists of 147 bp DNA, a tetramer of two histone 2As (H2As) and two H2Bs and two dimers of H3 and H4. The crystal structure (Figure 4b) has been solved indicating the electrostatic interactions and hydrogen bonds between the DNA and nucleosome [39]. Sequence specific DNA binding proteins are usually unable to recognise their binding site on such a contorted surface. The histone amino-terminal (NT) tails are accessible to the solute and can be post-translationally modified.

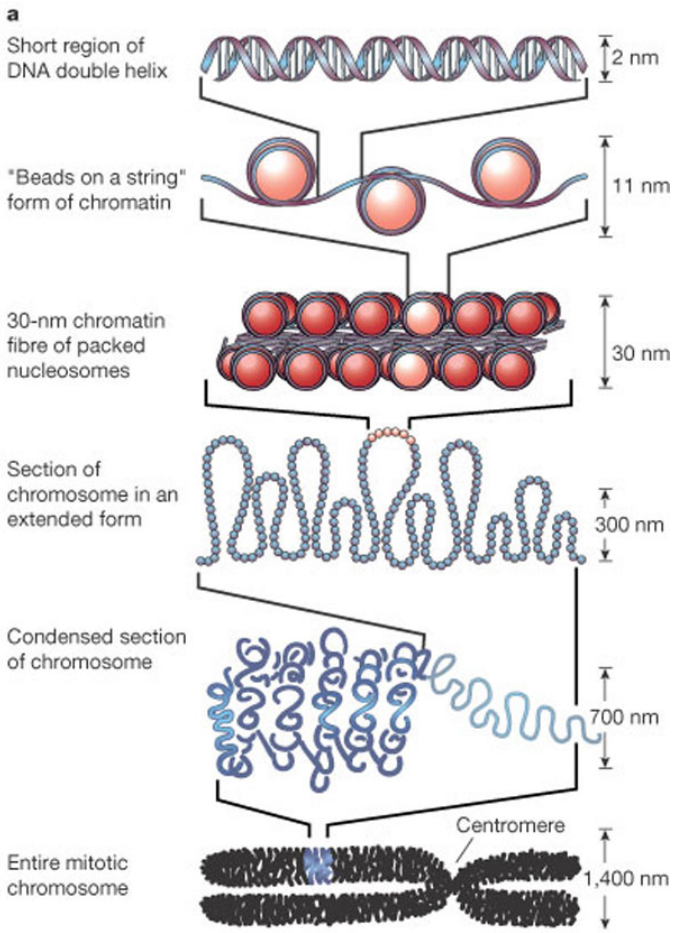

Figure 4. Chromatin. (a) DNA is wrapped around core particles to form nucleosomes that are packaged into higher order structures to ultimately form the metaphase chromosomes. (b) Nucleosome structure at 2.8Å. One DNA strand is in orange, the other green. The histones are in blue (H3), green (H4), yellow (H2A) and pink (H2B) as ribbon models. Picture taken from [3].

1.2.4 Histone structure and the histone code

The important function of core histones is indicated by their very high conservation among eukaryotic species. The core histones are small basic proteins sharing a similar

globular domain consisting of a helix-turn-helix motif. This domain allows dimerisation and interactions with DNA. Core histones also have a long, mainly unstructured NT "tail". Most histone tails (except H4) are not necessary for nucleosome assembly and 30 nM compaction [40], although they are the major sites of post-translational modifications (PTMs). This indicates that the NT tails are unlikely to *directly* regulate the structure of single nucleosomes. However, since histone tails may be able to regulate interactions between nucleosomes, or between nucleosomes and linker DNA, it may be that histone tails can regulate higher order chromatin structures. In this way they might also *indirectly* influence the structure and stability of single nucleosomes. Some of the globular parts of core histones encircled by the DNA in the nucleosome are also modified, leading to the possibility that these could *directly* affect the stability of single nucleosomes.

There are three ways in which nucleosomal structure can be modified and therefore the local accessibility of DNA to TFs (and other proteins) can be regulated. These are the selective use of histone variants (reviewed in [41]), PTM of histones and other chromatin proteins (reviewed in [42]) and ATP-dependent remodelling of nucleosomes (reviewed in [43]). Histone variants are often included in the chromatin at specialised chromatin structures or during specific signalling events in the cell. For example, H3.3 is incorporated into chromatin at active genes without the need for DNA replication [44] and macroH2A is found at higher than normal concentrations in the inactive X chromosome (of female mammals) [45].

It has been shown that regions of the genome that are actively transcribing generally contain more acetylated histones in the chromatin [46]. However, the simple model of "more acetylation, more transcription" is not accurate. To date, the following histone modifications have been found at specific sites: acetylation, ubiquitination, sumoylation and methylation on lysine residues, methylation on arginine residues, phosphorylation on serine and threonine residues, mono ADP-ribosylation on glutamic acid residues [47]. Many of these modifications are inter-dependent and can be present on histones in the cytoplasm or nucleus. It has been proposed that the pattern of these modifications form a histone code. This code might reflect the competence of the surrounding DNA sequences for various processes such as transcription and DNA repair.

In yeast it has been shown that H3 lysine 9 and 14 (H3K9 and H3K14) acetylation, H4 acetylation and H3K4 tri-methylation (me3) at transcriptional start

sites correlates with gene transcription [48]. The acetylation reduces towards the 3' end of the transcribed gene, indicating acetylation at these residues might be generally involved in transcription initiation events. Unlike H3K4me3, H3K4 di-methylation (me2) peaked in the middle of the coding sequences of the yeast genes and H3K4 mono-methylation (me) peaked at the end of the genes [48]. These results indicate the complexity of the histone code. Similar studies on human chromosomes have confirmed the results of the H3K4 methylation pattern found in yeast [49]. Many extensive ChIP on CHIP (chromatin immunoprecipitation followed by micro-array analysis of DNA isolated) studies of the human genome in different cells and under different conditions are needed to validate the histone code hypothesis and decipher the code properly.

Histone modifications can affect the accessibility of protein factors by two mechanisms (reviewed in [42]). The first is by regulating protein-protein interactions between modified histones and effector proteins such as transcriptional regulators. Chromodomains and bromodomains are protein domains that can recognise methylated and acetylated lysines respectively, and are present in many transcription cofactors (reviewed in [50, 51]). The second is to affect the protein-protein or DNA-protein contacts within and between nucleosomes, therefore affecting the stability of the nucleosomes and accessibility of effector proteins.

1.2.5 Higher order chromatin structure

Chromatin is the complex of DNA and protein, and functions to package the DNA in the nucleus in a regulated manner (reviewed in [3]). The nucleosomes pack together to form higher order structures, which fold up in an ordered way to form chromosomes. The dynamic structure of chromatin regulates DNA accessibility to protein factors involved in all forms of DNA metabolism, such as transcription, replication and DNA repair. A major mechanism of regulation of transcription is by altering the chromatin structure surrounding genes and their regulatory elements. By electron microscopy, an 11 nm fibre (looking like beads on a string) can be seen. This is thought to be the individual nucleosomes separated by linker DNA (between 10 and 80 bp long in humans). Chromatin 30 nM fibres are also seen, upon addition of linker H1, and this structure is thought to be packing of the 10 nM fibre by superhelical coiling or zigzagging (reviewed in [52]) (Figure 4).

There has been an explosion in whole genome analysis in recent years, with the aim of determining where nucleosomes are positioned on the genome in the living cell, which histone modifications the nucleosome contains, and how this correlates with transcription. These genome wide studies coordinate data from real time RT-PCR (which quantifies mRNA expression), and ChIP on CHIP studies. In yeast it has been shown that nucleosomes occupy DNA less densely at promoters of transcribed genes than at the promoters of inactive genes [48].

A model for further organisation of the chromatin by a series of loops involves the insulator DNA elements and associated protein complexes (reviewed in [53]). Individual insulator complexes might multimerise in such a way to cause the open "active" chromatin loops to surround a core of closed "inactive" chromatin. The insulator complexes could be anchored to the nuclear periphery. Matrix attachment regions (MARs) are also involved in higher order chromatin structure, by fixing specific A/T rich sequences to the nuclear lamina. MARs are only equivalent to Insulators in some cases. However the precise structure of chromatin compaction and regulation of the compaction is not known.

1.2.6 Heterochromatin and Euchromatin

Heterochromatin is a form of chromatin that is highly condensed throughout the cell cycle. The nucleosomes within heterochromatin have a slightly different structure to those in euchromatin, having a shorter linker region and longer region of protection from micrococcal nuclease [54]. The differences seen in nucleosome or higher order structure of the chromatin can be explained by the nucleosomal histones having different modifications, occurring as variants and being in complexes with other chromatin proteins. Heterochromatin histones (e.g. in the pericentromeric region and in inactive X chromosome) are generally less acetylated than euchromatin histones (reviewed in [55]). Methylation of H3K9 by SuVar39 is involved in heterochromatin formation. Heterochromatin protein 1 (HP1) binds to methylated H3K9 residues (reviewed in [56]). However, the code of histone modifications determining the state of heterochromatin is more complex. For example H3K9 trimethylation and H3K27 monomethylation is found to be more abundant in pericentromeric heterochromatin than euchromatin (which contains mono or dimethylated H3K9 residues at silenced regions).

Telomeric heterochromatin in *S. cerevisiae* is silenced by Repressor activator protein 1 (RAP1) binding to telomeric repeats, recruiting large complexes including the Silent information regulator (SIR) proteins. One SIR protein is a histone deacetylase (HDAC), which probably marks the histones in the area for transcription repression (reviewed in [56]). Often telomeric complexes and other heterochromatin domains are found near the nuclear periphery indicating that there may be a relationship between transcriptional activity, and nuclear position [57]. Heterochromatin formation is often associated with RNA containing complexes. For example, during development one of the X chromosomes in female mammals is randomly and heritably inactivated, by formation of a heterochromatin state dependent on a special RNA, called Xist (reviewed in [58]).

1.2.7 Transcription factories

The chromosomes are positioned in precise locations in the nucleus, surrounded by nuclear sub-organelles interconnecting the molecular processes of gene expression (reviewed in [59]). There are thousands of sites where active transcription occurs in the nucleus called transcription sites **(**factories). These sites in the nucleoplasm are sometimes close to nuclear speckles (which contain mRNA processing proteins) probably due to the connection between mRNA splicing and transcription. In G1 phase only, some transcription factors are localised only in 1-3 sites called OPT domains (Oct-1/PTF/transcription) and these sites do not contain factors for RNA processing [60]. Transcription factories are places where active transcription of different genes co-localise, resulting in the hypothesis that upon activation, a gene migrates to the transcription machinery in the transcription factory [61]. The dynamic transcription factories and other nuclear structures can regulate gene expression at the level of transcription and mRNA processing events [62]. De-regulation of the nuclear features can lead to de-regulation of gene expression and diseases such as cancer [63]. Protein complexes the size of spliceosomes can move through the nucleus in minutes by diffusion, indicating an active system of transport for nuclear proteins may not be necessary [64, 65].

1.2.8 Nuclear Structure

Within the nucleus there are many subcompartments with specialised functions (Figure 5).

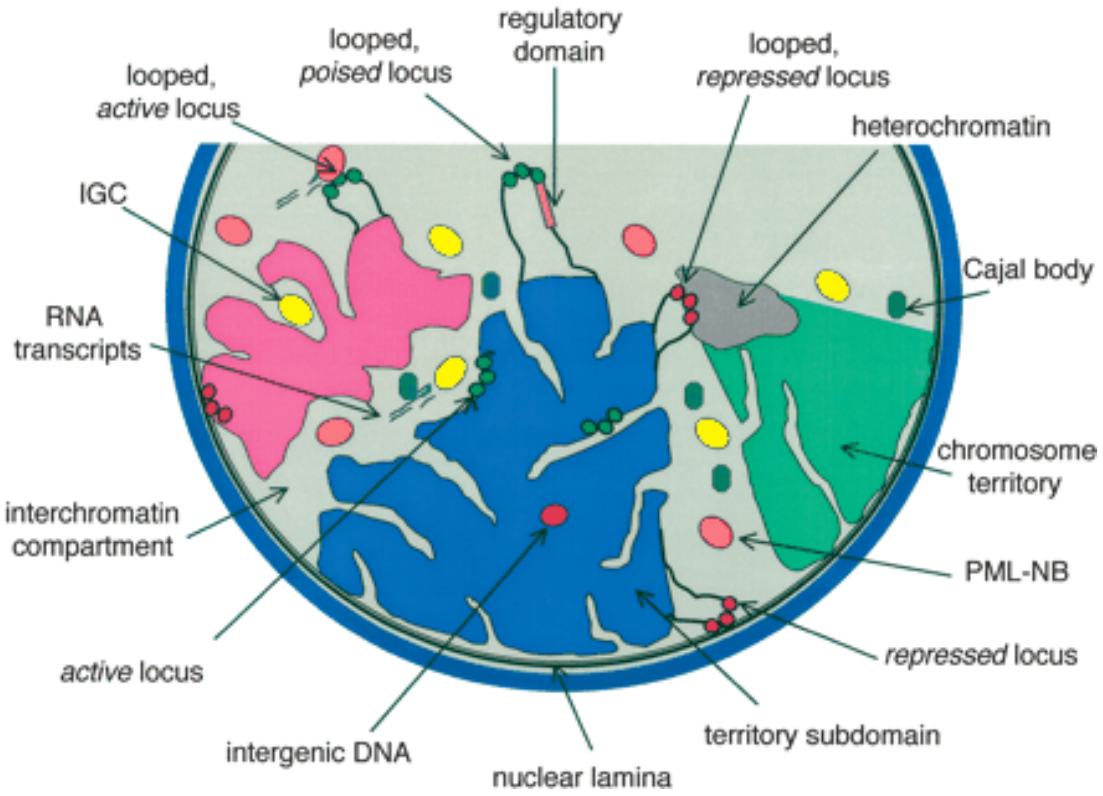

Figure 5. Nuclear Organisation of gene activity and silencing. Nuclear compartments involved in gene expression are shown. Each feature could be involved in allowing or preventing access of TFs such as NF-κB to genes. In the text chromosome territories, heterochromatin, nuclear laminar are described. The nuclear bodies involved in transcription, or gene regulation are PLM bodies, interchromatin granule clusters (IGCs) and cajal bodies. PML bodies are a target of virus infection and there are 10-30 per nucleus. PML bodies are involved in transcription regulation since they contain TFs and chromatin modifying, although actively transcribing RNAPII was not found [67].There are 1-10 dynamic Cajal bodies per nucleus that contain pre-mRNA splice factors. It is thought that small nuclear ribonucleoprotein particles (snRNP) biosynthesis and trafficking occur there, and are then moved towards the speckles. Gems (Gemini of cajal bodies) are next to or overlapping with cajal bodies and could be involved in snRNP maturation. Cleavage bodies are found diffusely and in 1-4 foci (next to or overlapping with cajal bodies) and contain Cleavage stimulation factor and cleavage and polyadenylation proteins. IGCs are probably a store of proteins for mRNA processing, and transcription is tightly coordinated with mRNA processing. The model is therefore that actively transcribing genes often found close to these nuclear bodies. Picture taken from [26].

Genes are preferentially located on chromosome territory surfaces whereas intergenic regions are buried within the chromosome territory. Transcription occurs on the invaginated surface of the chromosomes, on the interface with the interchromatin compartment containing many nuclear bodies involved in regulation of gene expression (reviewed in [26] [65]). The positioning of chromatin territories, actively transcribing loops and nuclear bodies can regulate the pattern of gene expression by altering the access of transcription factors to the chromatin, the RNA processing enzymes to the nascent RNA, and the processed RNA to the nuclear pores.

A fluid double membrane surrounds the nucleus. On the inner surface of the nuclear membrane is the peripheral nuclear lamina. It consists of lamin A/C and B proteins, which link chromatin to the edge of the nucleus (reviewed in [26] [65]). Location of a gene near the nuclear periphery is thought to be an indication that the gene is repressed (reviewed in [26]). The repression is not necessarily due to heterochromatin formation. For example, the silent immunoglobulin heavy chain (IgH) locus is not associated with heterochromatin [66].

1.3 Regulation of Transcription

1.3.1 Transcription Factors (TFs)

TFs coordinate cellular signals to regulate transcription and therefore gene expression [1]. The basic model is that stimuli activate signal transduction pathways to activate TFs that can then bind to enhancers or proximal promoters to regulate transcription. TFs regulate transcription at all the different steps in the transcription cycle, especially the early step of PIC formation. Transcription can be positively regulated (transcription activation) or negatively regulated (transcription repression) (reviewed in [68]). Activation requires the generation of an accessible DNA template and transcription promoting protein-protein interactions (e.g. between TFs and GTFs). Repression can be thought of as the generation (or maintenance) of a stable repressive chromatin structure or by prevention of the transcription promoting protein-protein interactions.

Transcription activation by enhancer bound transcription factors is thought to be through recruitment of GTFs or transcription co-activators (Section 1.3.2) *to the core promoter* via looping of the DNA (Figure 6, left). However, CHIP experiments have shown that RNAPII is complexed with many enhancers before the core promoters (reviewed in [36]). In these cases, the enhancer bound TFs recruit GTFs and co-activators *to the enhancers*. Gene specific transcription occurs after looping of the DNA and RNAPII binding to the promoter (Figure 6, right). It is likely that both situations represent specific cases at different promoters. This could be due to the order of promoter versus enhancer recruitment to GTF/RNAPII containing transcription factories. The order of cofactor recruitment is discussed further in Section 1.3.2.

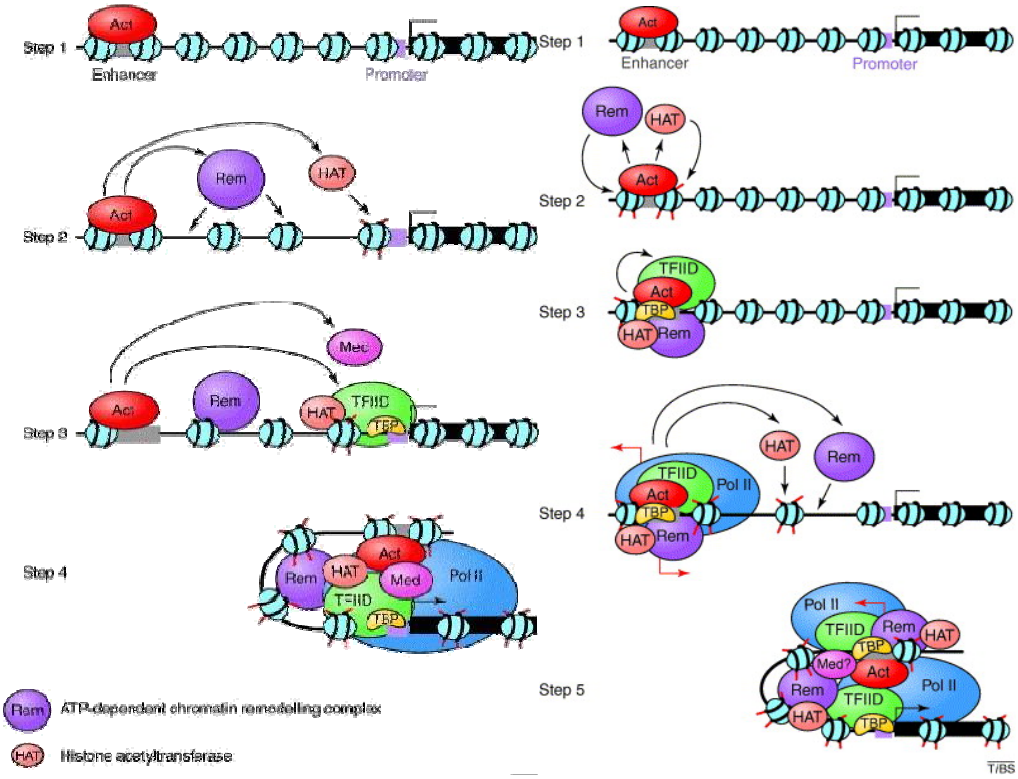

Figure 6. Two models for enhancer bound transcription factor transactivation. Transcription factors (Act) bind to enhancer regions and recruit nucleosome remodellers (Rem), histone acetyltransferases (HAT) and GTFs directly to the promoter (left picture) or first to the enhancer (right picture). The GTFs shown are TFIID, TBP, Mediator (Med) and RNAPII (pol II). Picture adapted from [36].

Many TF binding sites are free from positioned nucleosomes, providing easy access for the activated TF to bind to DNA. However, when DNA binding sites are not easily accessible due to positioned nucleosomes and varying degrees of nucleosome stability and higher order compaction, TFs can often still function. The mechanism of how TFs do this is dependent on each gene and transcription factor, but can involve:

I. Positioned nucleosomes. Although the DNA of nucleosomes is not normally able to bind TFs, nucleosomes are not always repressive to TF binding. Many reports indicate that various TFs can bind to arrays of nucleosomal DNA. Often the positioning of nucleosomes is critical for the transcriptional potential of the gene (reviewed in [69]). This is because the TFs can sometimes bind the linker DNA, or at the edges of the nucleosomes (where transient disassociation of histone-DNA interactions occur [70]) or on the outer surface of the DNA as it coils around the histone tetramer.

II. Cooperativity between TFs. Most eukaryotic genes are regulated by many TFs, which activate transcription synergistically. This can be due to the weak binding of one TF to nucleosomal DNA enhancing the affinity of another TF to bind the DNA through TF-TF interactions or distortion of the histone-DNA interactions.
III. Dynamic and flexible nucleosomes. Despite the many contacts between histones and DNA in the nucleosome, there is strong evidence that nucleosomes are not static structures, and the contacts are dynamic, allowing access to DNA even within the nucleosome [71].
IV. Histone exchange. It is possible that some TFs bind to DNA during transient periods of histone exchange. For example, during S phase of the cell cycle, histones are deposited onto the newly synthesised strand of the DNA. Some TFs could remain closely associated with chromatin and bind to their cognate sequences as they emerge from the replication machinery.

1.3.2 Transcription cofactors

Transcriptional cofactors are defined as proteins that regulate transcription without directly binding to DNA sequences. Like transcription factors, the transcription cofactors can either activate or repress transcription by regulation of DNA accessibility, or by forming a bridge between TFs and GTFs or RNAPII (reviewed in [72]). Transcriptional cofactors normally fulfil their functions when part of multi-protein complexes, and some proteins are found in many different complexes. The cofactor complexes that are involved in regulating chromatin accessibility contain enzymatic subunits (reviewed in [73]). Histone acetyltransferases (HATs) acetylate the lysine groups of histone and non-histone proteins. Histone deacetylases (HDACs) remove the acetylation. Histone methyltransferases (HMTs) methylate arginines and lysines of histones and sometimes non-histone proteins. ADP-ribosyltransferases also modify histones and non-histone proteins. ATP-dependent nucleosome remodelling factors contain subunits of the SNF2 ATPase superfamily of proteins and are involved in the re-positioning of whole nucleosomes.

Complex	Mechanism	↑ or ↓	Subunit	RelA/p65?
p300/CBP	bridges and HAT	↑ mainly	p300/CBP	Yes
PCAF	bridges and HAT	↑	PCAF (GNAT family)	Yes
SAGA	bridges and HAT	↑	hGCN5L (GNAT family)	Yes? TAFs
TFTC	bridges and HAT	↑	hGCN5L (GNAT family)	Yes? TAFs
TFIID	bridges and HAT	↑	TAF1 (HAT) and TAFs	Yes
STAGA	bridges and HAT	↑	hGCN5L (GNAT family)	Yes? TAFs
SRC-1	bridges and HAT	↑	various:e.g. CBP, PRMT1	Yes
SRC-2	bridges and HAT?	↑	various:e.g. CBP, PRMT1	Yes
SRC-3	bridges and HAT	↑	various:e.g. CBP, PRMT1	Yes
tip60	HAT	↑ and ↓	tip60 (MYST family)	Yes
MAF2	HAT	↑	MOF (MYST family)	
AML1	HAT	↑ and ↓	MOZ (MYST family)	
MORF	HAT	↑ and ↓	MORF (MYST family)	
HBO1	HAT	↓	HBO1 (MYST family)	
TFIIIC	HAT	↑	one of three TFIIIC proteins	
Elongator	HAT	↑	Elp3	
CIITA	HAT	↑	CIITA	
hBRM	remodeller	↑	Brm (SNF2 subfamily)	
hBRG	remodeller	↑	Brg (SNF2 subfamily)	Yes
hBAF	remodeller	↑	Baf (SNF2 subfamily)	
RSF	remodeller	?	hSNF2h (ISWI subfamily)	
hACF/WCRF	remodeller	?	hSNF2h (ISWI subfamily)	
hCHRAC	remodeller	?	hSNF2h (ISWI subfamily)	
hWICH	remodeller	?	hSNF2h (ISWI subfamily)	
NoRC	remodeller	?	hSNF2h (ISWI subfamily)	
NuRD	remodeller/HDAC	↓	Mi-2, HDAC1/2 (CHD1 subfamily)	
N-CoR	HDAC	↓	HDAC1/2 and/or HDAC3 (class 1)	Yes
SMRT	HDAC	↓	HDAC1/2 and/or HDAC3 (class 1)	Yes
co-REST	HDAC	↓	HDAC1 and/or 2 (class 1)	
sin3-SAP	HDAC	↓	HDAC1 and 2 (class 1)	
sir2	HDAC	↓	sir2p (class 3)	SIRT1
PRMT1,2,4	HMT (arginine)	↑	PRMT1,2,4	PRMT1/4
PRMT5	HMT (arginine)	↓	PRMT5	
SET	HMT (lysine)	↑ and ↓	various	
Mediator	bridges	↑ and ↓	? Nut1	Yes
PARP1	ADP-ribosyltransferase	↑ mainly	PARP1	Yes

Table 1. Common cofactors of transcription. Columns: Complex is the name of the cofactor complex. Mechanism is whether the cofactor regulates transcription by interaction with TFs and GTFs or pol II (bridges), or by the indicated enzymatic activity. ↑ or ↓ or ? indicates whether the complex activates or represses transcription or its effect is unknown. Complexes in bold are capable of repression. Subunit indicates which protein subunits have enzymatic activity. RelA(p65)? indicates which complexes or subunits are involved in regulation of RelA(p65) dependent transcription. The HATs and remodellers are grouped in families according to sequence homology. SNF2 subfamily: large (2MDa) complexes, bromodomain and ATPase domain, destabilise/disrupt nucleosomes. ISWI subfamily: smaller (500-800 kDa) complexes, SANT domain and ATPase domain, stabilise/assemble nucleosomes. CHD1 subfamily: mid-size (1-1.5 kDa) complexes, chromodomain and DNA binding motif and HDAC activity. HDAC classes are according to homology to the yeast proteins Rpd3 (class 1), Hda1 (class 2), Sir2 (class 3). Class 1 proteins are ubiquitous and nuclear, while class 2 proteins are tissue specific and some are cytosolic proteins. HDAC activity of Class 3 is by an alternative NAD+ dependent mechanism. References used [74-79].

Common human transcriptional cofactor complexes are listed in Table 1, which indicates the mechanisms they use, their effect on transcription (positive or negative), their main enzymatic subunits and whether they are known to be involved in

transcription regulation by the main NF-κB family member, RelA(p65). Further details for the RelA(p65) cofactors are described in Section 2.6. Yeast homologues of many of these cofactors exist, but these are not included in the table.

From Table 1 it is evident that the same complex can activate or repress transcription, depending on the experimental system. This means it is sometimes difficult to label a cofactor of transcription as a co-activator or co-repressor. Many cofactors can interact with the same transcription factor to coordinate the regulation of a specific gene. An example of how this is achieved temporally is exemplified by extensive ChIP studies performed on the estrogen receptor responsive gene pS2 gene [80]. This study illustrates common themes in the current thinking of cofactor recruitment (reviewed in [73]):

I. Different cofactor complexes can be recruited to the gene in an ordered manner, and assist the function of others.
II. The recruitment of a specific cofactor complex may be cyclical.
III. Different transcription factors recruit different cofactor complexes.
IV. The same transcription factor may recruit different cofactor complexes at different gene control regions.
V. The order of cofactor recruitment may depend on the TFs present, the promoter and enhancers of the gene, and the chromatin status around the gene.

Regulation of transcription can also occur by RNA mediated mechanisms. Recently, it has been shown that duplex RNA targeted to promoter regions of specific mammalian genes can repress transcription and gene expression of that gene. The mechanism of the process is not fully characterised, and only sometimes involves CpG island methylation (reviewed in [81]).

2 NF-κB dependent Transcription

2.1 The Mammalian NF-κB family members

NF-κB proteins were given their name as they were first identified as nuclear factors that could bind to a kappa (κ) immunoglobulin light chain enhancer from mature B cells [82]. The NF-κB family of inducible transcription factors has seven members, RelA(p65), c-Rel, RelB, p50, p52, p105 and p100 (reviewed in [83]). All family

members share a conserved Rel Homology Domian (RHD), which is involved in DNA binding, dimerisation between family members and interaction with the inhibitor of NF-κB (IκB) family of proteins. p100 and p52 are coded by the NF-κB2 gene and p100 is inducibly proteolytically cleaved to form p52. p105 and p50 are coded by NF-κB1 gene and p105 is constitutively proteolytically cleaved to form p50. RelA(p65), c-Rel, RelB, p50 and p52 proteins homo- and heterodimerise to form active and inactive transcription factors. Most possibilities of heterodimers and homodimers of NF-κB have been identified, apart from RelB that does not homodimerise or heterodimerise with RelA(p65) or c-Rel. However, the dimers are formed with different affinities, where the RelA(p65) and p50 have a particularly high affinity to dimerise. The dimers are complexed with IκBs in the cytoplasm of most cell types. The inhibitors share homology with the amino terminal (NT) of p100 and p105, which also inhibit NF-κB function. Figure 7 shows the domain structure and important features of the NF-κB and IκB family of proteins.

2.2 Signal transduction pathways

There are many signals that lead to activation of NF-κB members, and transcription of response genes. Activating signals include bacterial, viral and parasite products, cytokines, stress conditions, antigens and mitogens, indicating NF-κB is activated in response to infection, inflammation and cellular stress (reviewed in [84]). The simple view of NF-κB regulation is that NF-κB is normally held inactive in the cytoplasm by IκB proteins, and activation through various signal transduction pathways leads to IκB degradation and the nuclear accumulation of specific NF-κB molecules. NF-κB then binds to consensus sequences to activate target gene expression. Termination of NF-κB activation occurs due to negative feedback (since activated NF-κB upregulates IκBα expression) [85] as well as ubiquitin mediated proteosomal degradation of NF-κB [86].

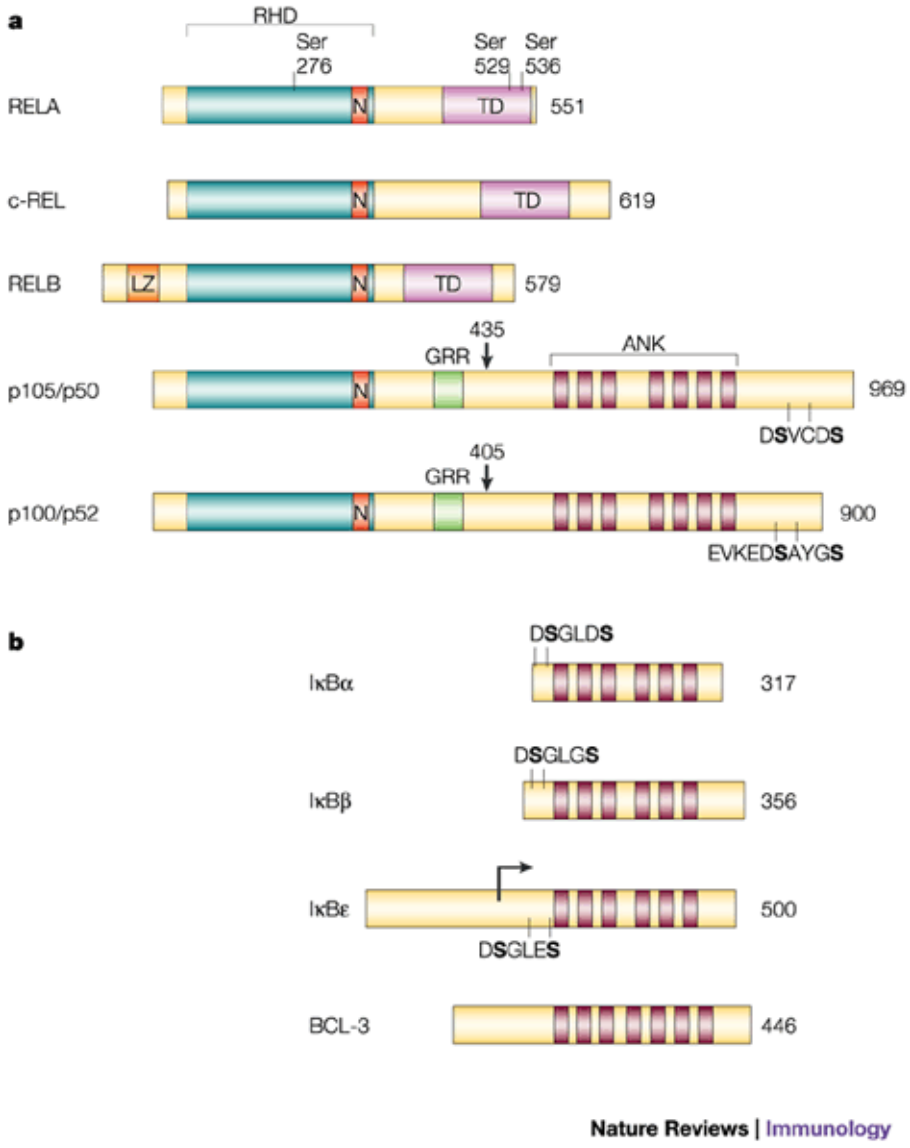

Figure 7. The NF-κB and IκB proteins. This figure shows the domain structure of (a) the NF-κB and (b) the IκB family membezs. All NF-κB family members share a Rel Homology Domain (RHD) and a nuclear localisation signal (N). RelA (also called p65), c-Rel and RelB contain non-homologous C-terminal transcription activation domains (TD). The ankaryin repeats (ANK) domains (protein-protein interaction domains) are shown. The inducible phosphorylation sites required for IκB degradation are shown in bold. The glycine rich region (GRR) and the phosphorylation sites required for C-terminal processing of p100 and p105 are shown. The proteolytic cleavage site is shown with an arrow. The leucine zipper (LZ) of RelB is shown. Three important inducible phosphorylation sites of RelA(p65) are shown which enhance the transcription potential of NF-κB. Picture taken from [83].

It is now thought that before stimulation NF-κB/IκBα complexes shuttle between the nucleus and cytoplasm, whereas after stimulation, and IκB degradation, the NF-κB complex is mainly found in the nucleus [87]. RelA(p65) and p50 each have a nuclear localisation (NLS), but when bound to IκBα, the NLS of RelA(p65) is masked [88]. Therefore, before stimulation the equilibrium of the NF-κB/IκBα complex is towards cytoplasmic localisation due to the complex containing one efficient nuclear export signal (NES) of IκBα and only one NLS (of p50). Upon stimulation and degradation of IκBα, the NES is lost, an accessible NLS (of RelA(p65)) is gained and therefore nuclear accumulation of NF-κB occurs. For non-stimulated NF-κB complexes containing IκBβ or ε, the mechanism for the bulk cytoplasmic retention and stimuli dependent nuclear accumulation of NF-κB is slightly different. IκBβ containing NF-κB complexes show no pre-stimuli nucleo/cytoplasmic shuttling since IκBβ does not contain an NES, and IκBβ binding to NF-κB masks both the RelA(p65) and the p50 NLS [89]. There is also some evidence that the main role of IκB proteins in NF-κB inhibition is not by cytoplasmic sequestration, but by another mechanism suppressing the basal transcription of NF-κB genes [90]. Support for this hypothesis comes from the observation that GFP-p65 is nuclear when overexpressed, but when a DNA binding deficient mutant is used it is cytoplasmic [90]. However, further investigations are needed to clarify the precise role of the IκB proteins in cytoplasmic sequestration of NF-κB.

There are two main signalling pathways that activate NF-κB nuclear accumulation (reviewed in [91]). In the classical pathway, pro-inflammatory cytokines and other stimuli activate the tumour necrosis factor (TNF) receptors, Toll like receptors and interleukin 1 (IL1) receptors. This leads to activation of the IκB kinase (IKK) complex and IKKβ dependent phosphorylation of IκB at positions corresponding to serine 32 and serine 36 in IκBα. Phosphorylation of IκB results in lysine ubiquitination (lysine 21 and 22 in IκBα) and subsequent degradation by the 26S proteosome. The classical pathway leads to activation of RelA(p65)/p50 heterodimers, as well as RelB and c-Rel containing dimers and is important for expression of proteins involved in innate immunity (Figure 8a). There are many variations of the classical NF-κB pathway, with multiple signals all eventually leading to activation of the IKK complex (Figure 8a).

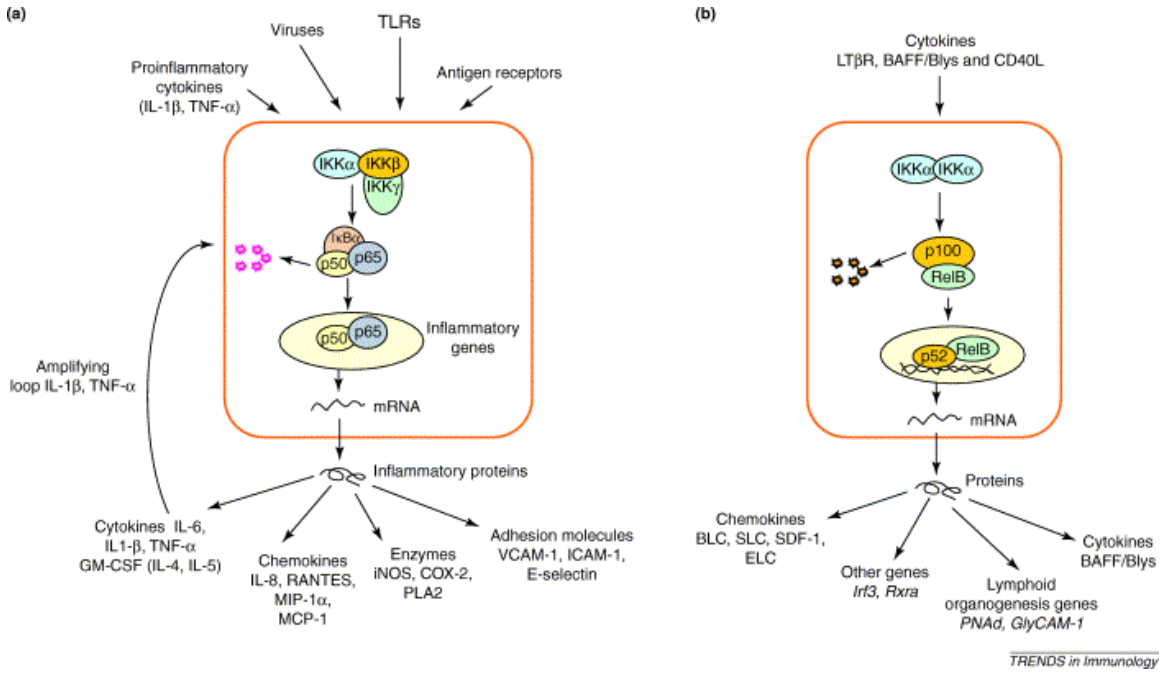

Figure 8. The classical and an alternative pathway for NF-κB activation. (a) Multiple signals such as cytokines, viruses, toll like receptor ligands and antigens activate the classical pathway. IκBα (or β) is degraded, and NF-κB (here RelA(p65)/p50 heterodimers) translocates to the nucleus to activate transcription of genes involved in innate immunity. (b) An alternative pathway, activated by specific cytokines, leads to IKKα dependent phosphorylation of p100. Proteolytical cleavage results in p52/RelB heterodimers entering the nucleus and activating transcription of genes involved in adaptive immunity. Picture taken from [91].

An alternative pathway is important for adaptive immunity (mediated by T and B cells) (Figure 8b). The response of fully developed T and B cells to chemokines is mediated through various receptors such as BAFF, LTβR, and CD40. Cytokine stimulation of these receptors leads to IKKα phosphorylation of p100 and the processing of p100 to p52. This results in the nuclear translocation of RelB/p52 and activation of specific genes. Other alternative NF-κB activating pathways have also been observed. For example, endoplasmic reticulum stress or amino acid starvation leads to the dissociation of IκB-NF-κB but not degradation of IκB. The activation requires to phosphorylation of a translation elongation factor (eIF2α) [92].

2.3 Signal specificity of the NF-κB response

Many signals activate NF-κB, through many different signalling pathways. Figure 8 describes two major pathways that explain how genes involved in the innate immune

response can be regulated separately from genes involved in the adaptive immune response. However, there are more layers of specificity since one signal will activate only a small subset of specific genes. This section describes the layers of regulation, which could activate NF-κB dependent transcription of specific genes.

2.3.1 Degradation of specific IκB proteins

Some signals, or combination of signals lead to degradation of specific IκBs. For example, in 70Z/3 pre-B cells lipopolysaccharide (LPS) and IL1 could induce degradation of IκBα and IκBβ whereas only IκBα was degraded upon phorbal 12-myristate 13-acetate (PMA) stimulation [93]. This leads to differences in the kinetics of the responses, since IκBα is involved in a quick but transient NF-κB response whilst IκBβ controls a slower, more prolonged response. Also, specific IκB proteins can associate preferentially with some dimers. For example IκBε complexes mainly with c-Rel and RelA(p65) [94]. The degradation of specific IκB proteins can then lead to the nuclear accumulation of specific NF-κB dimers.

2.3.2 Activation of transcription by specific NF-κB dimers

It has been shown that some signals lead to binding of specific dimers at particular promoters, leading to differential regulation of specific genes. For example, it was found that upon LPS stimulation RelA(p65) is recruited to the IL8 promoter, whereas c-Rel is recruited to the IL-12p35 promoter in dendritic cells [95]. At the ECL and MDC promoters, first RelA(p65) was detected to bind after LPS stimulation, and then RelB, leading to RNAPII recruitment. At the IL-12p40 promoter a similar exchange of RelA with RelB occurred after LPS stimulation but with disassociation of RNAPII and decrease in mRNA synthesis [95]. The exchange of NF-κB dimers was not seen with TNFα stimulation at the ECL promoter, and this indicates that there can be a stimuli specific exchange of NF-κB dimers on the promoter resulting in modulation of transcriptional activity [95]. Different promoters have been shown to be dependent on specific NF-κB proteins for activity, although the sequence of the κB site does not correlate with the ability of specific dimers to regulate the particular gene [96].

2.3.3 Cooperation with other TFs and cofactors

Some signals can activate NF-κB, as well as activate or repress other TFs or cofactors that function with NF-κB to activate, or repress particular genes. Activation of a single TF may not lead to activation of a gene that requires coordinated activation of many TFs. Therefore, only appropriate stimulation would lead to activation of the TFs involved and transcription of the gene. An example of this kind of cooperativity between NF-κB, IRF1, c-Jun and HMGI(Y) at the interferon beta (IFNβ) enhanceosome is discussed in Section 2.7 (reviewed in [97]).

2.3.4 Post translational modifications (PTMs)

Some signals release IκB as well as induce PTMs of NF-κB or cofactors, which could result in the regulation of specific genes. RelA(p65) has been shown to be inducibly phosphorylated, acetylated, ubiquitinated and prolyl-isomerised (reviewed in [98]). The analysis of the effect of the PTMs is normally limited to its effect on general transcriptional ability of RelA(p65). For example, protein kinase A (PKA) mediated phosphorylation of RelA(p65) at serine 276 leads to enhanced transactivation, due to increased interactions with p300 and cyclic AMP response element binding protein (CBP) co-activators [99]. However, signal specific PTMs of RelA(p65) or cofactors could also result in the regulation of specific genes.

2.4 κB binding in vitro and in vivo

NF-κB dimers bind to the consensus DNA sequence of the κB site GGGRNNYYCC (N= any, R= purine A/G, Y= pyrimidine C/T). The κB consensus sequence is degenerate since different dimers bind to slightly different sequences with different affinities *in vitro*, and specific NF-κB dimers can bind to many variations of the consensus κB sites (reviewed in [100]). The crystal structures of NF-κB (RelA(p65)/p50)) bound to κB sites show some structural features that may explain the ability of NF-κB to bind to different DNA sequences [88, 101]. The RHD consists of two β-sheet immunoglobulin-like folds. The NT β fold makes base specific contacts with the major groove using amino acid residues on flexible loops extending from the rigid β-sheet immunoglobulin folds. The C-terminal (CT) β fold makes hydrogen bonds and hydrophobic interactions with the CT β fold of the other subunit, forming strong bonds between the two subunits of the dimer. However, this CT β fold

only interacts with the phosphoribose backbone of the DNA, and does not make base specific contacts. Since there is some flexibility between the two β folds of the RHD this may explain why there is flexibility in the consensus κB sequence.

Although different homodimers showed different affinities to different κB sites *in vitro* [102], it is currently difficult to predict which NF-κB dimer will bind to a particular κB site *in vivo*. It has been shown that the sequence of the κB site does not correlate with the ability of some specific dimers to regulate particular genes [96]. However, certain κB sites are thought to bind only selective dimers. For example, RGGAGAYTTR is in the promoter regions of some genes activated by the alternative pathway (Section 2.2, page 27) and has been shown to bind only the RelB/p52 heterodimer [103]. The κB sites in many promoters are conserved through evolution indicating that the sequence has an important function for selectivity. Experiments using lentiviral-based κB reporters in different NF-κB knock out cells indicated that the κB sequence does confer the requirement for specific NF-κB subunits to be present in fibroblasts for transcriptional activity [104]. However, the κB sequence does not primarily determine which NF-κB dimer binds, instead it determines which NF-κB dimer can activate transcription by forming a productive interaction with signal specific cofactors [104].

It is predicted that NF-κB would not be able to bind a κB site if it was tightly packed into a nucleosome due to steric hindrance (reviewed in [105]). However, it has been shown that *in vitro* p50 homodimers can bind to a nucleosomal κB site, without disturbance of histone-DNA contacts [106]. Whether this could also be the case for p50 homo- or heterodimers on chromatin *in vivo* is unknown. It would be expected that not only the presence of nucleosomes would effect the NF-κB binding to κB sites, but also the "status" of nucleosomes and histones. ChIP have shown that some promoters containing κB sites are more accessible to NF-κB binding than others. LPS induced the binding of RelA(p65) to some promoters more quickly than to other promoters in Raw (macrophage) cells [107]. The promoters that could recruit RelA(p65) quickly (20 min) had a low level of constitutive H4 acetylation, whereas the promoters that recruited RelA(p65) slowly (approx 1 hour 30 min) did not [107].

One extensive ChIP on CHIP study found 209 TNFα inducible RelA(p65) binding sites on the non-repetitive regions of chromosome 22 in HeLa S3 cells [108].

Approximately 30% of the sites were within 5 kbp from the 5' end (ATG) of an annotated gene and 40% of binding sites were found in intronic regions. Only half of the bound fragments contained a consensus κB site indicating that this is not always a requirement for NF-κB chromatin association.

2.5 Transcription activation domain (TAD) of RelA

Transcription activation domains (TADs) of TFs are grouped into classes, although many TADs contain elements of more than one class (reviewed in [109]). The classes are formed according to amino acid composition and are acidic, proline, glutamine or serine/threonine rich. TADs are thought to activate transcription by forming interactions with cofactors and GTFs. How this is achieved is not clear, due to the lack of intrinsic stable structures of most TADs. However, many TADs (including the TAD of RelA(p65)) can be induced to form structures under certain conditions. RelA(p65) contains two acidic transcription activation domains (TA1 and TA2) with conserved repeated amino acid motifs [110, 111]. Together they make the transcription activation domain (TAD). TA1 consists of the last 30 amino acids of RelA(p65) (amino acids 521-551), and TA2 consists of amino acids 441-521. They were identified by performing transient transfection experiments using a GAL4 driven chloramphenicol acetyl transferase reporter and the DNA binding domain of GAL4 (GAL4-DBD) fused to different deletion mutants of RelA(p65) [110]. Mutagenesis indicates that important features of TA1 are the acidity and position of the repeated motifs in a modelled potential α-helix. A model of the α-helical motifs is shown in Figure 9.

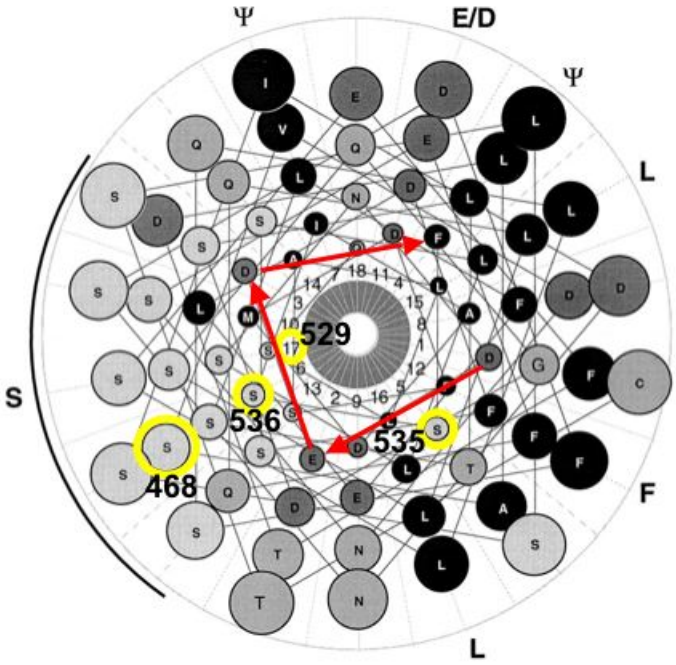

Figure 9. The modelled helix of the conserved repeated motifs in RelA(p65) TAD. The repeated motifs are:
Inner circle : TA1 from human/mouse (531-549) DEDFSSIADMDFSALLSQ
2nd circle: TA1 from Xenopus (508-525) GDSLTSLFELDFSSLLSN
3rd circle: TA2 from Human/mouse (463-480) FTDLASVDNSEFQQLLNQ
Outer circle: TA2 from Xenopus (430-447) CTSLSSIDNSDFSQLLSE
The red line shows the path of the TA1 from human or mouse, and the yellow circles indicate the positions of serines found to be phosphorylated in the human TAD. Picture adapted from [111].

Nuclear magnetic resonance showed that the TAD (428-551) is unstructured (random coil) in solution [112]. Circular dichroism measurements of TA1 (521-551) confirmed that the TA1 is normally unstructured. However, addition of a hydrophobic compound, resulted in an α-helical structure (modelled in Figure 9). This indicates that if the amino acids in TA1 are protected from water interactions, as could happen in a large protein complex, then it could form an α-helical structure, and make specific protein-protein interactions. The transcription activation domain of VP16 is also an unstructured acidic domain, and like RelA(p65)-TAD, the VP16-TAD can interact with TFIIB and TFIID components [113]. Interestingly, VP16-TAD was shown to form an α-helix upon binding to TAF9 ($TAF_{II}31$). VP16-TAD interaction with TAF9 was dependent on the hydrophobic residues that are also conserved in the repeated motifs in RelA(p65)-TAD [114]. Many cofactors are known to interact

directly with the RelA(p65)-TAD; these are p300 [115], TFIIB, TBP, $TAF_{II}250$, $TAF_{II}80$, $TAF_{II}28$ [116, 117], $TAF_{II}105$ [118], Silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) [119] and the Mediator complex [120].

2.5.1 Phosphorylation of RelA(p65)-TAD

The TAD of RelA(p65) has been found to be phosphorylated at many residues, as described in Table 2. The phosphorylation of the TAD was mainly shown to activate transcription. Three of the five phosphorylation sites occur in the serine rich region of the α-helical model of the TAD motifs (Figure 9). Phosphorylation of these residues would increase negative charged patches to form on one side of the helix. This could alter the interaction surface available to proteins, and explain the differences in interaction partners of TAD upon phosphorylation.

Site	Stimuli	Kinase	Pathway	Cells tested	Function	Ref
Ser536-P (TA1)	IL-1	IKKα/β p38	PI3K/Akt	NIH3T3, 293	Transcription from reporter increased, cooperation with p300/CBP	[121-123]
	TNFα	IKKα/β (cytosol)	TRAF2,5 and TAK	HeLa, 293 MEFs		[124, 125]
	LPS	IKK2		Monocytes, macrophages	Activates reporter	[125, 126]
	Lymphotoxin-β receptor activation	IKK1		HT29 cells	Activates reporter	[127]
	PMA + A23187			Jurkat		[125]
	doxorubicin and etoposide	RSK1 (nuclear)	p53 activation	Saos 2	Activates by reduced RelA/IκB interaction	[128]
	PMA + ionomycin	cytosol		Jurkat T cells		[129]
Ser529-P (TA1)	TNFα	Casein Kinase II	cAMP	HeLa	Transcription from reporter increased	[130]
Ser535-P (TA1)		CaMKIV	Maybe MAPK	HeLa	Transcription from reporter activated, CBP interaction	[131, 132]
Ser468-P (nucleus) (TA2)	PMA + ionomycin	IKKε		Jurkat T cells	Transcription from reporter increased	[129]
	TNFα	IKKβ		Various	No effect detected	[133]
	IL1	IKKβ		Various	No effect detected	[133]
		GSK-3β		fibroblasts	Possible repression of basal level	[134]
?Thr505-P			ARF, ATR		ARF represses transcription, via HDAC1	[135]
?-P (TA2)	PMA			HeLa	Transcription from reporter increased	[111]

Table 2. Phosphorylation sites in RelA(p65) TAD. Columns: Site indicates the known phosphorylation sites, unsure sites are indicated with a question mark (?). When identified other features are listed in the columns. A blank column indicates an unknown feature. Ser stands for serine, thr for threonine, P for phosphorylation.

2.6 Known Cofactors of RelA(p65) dependent Transcription

Hundreds of different proteins regulate the NF-κB response. The regulation normally involves IκB degradation and nuclear import of NF-κB. However, NF-κB dependent transcription is also regulated once inside the nucleus, by cofactors of transcription. Some proteins activate NF-κB dependent transcription (co-activators), while other

proteins repress transcription (co-repressors). However, proteins that are usually co-activators of transcription can also act as co-repressors under some specific circumstances, and *vice versa*.

2.6.1 TBP, TAFs, PC4 and TFIIB

TBP and TFIIB are general transcription factors involved in accurate transcription initiation by RNAPII. Both proteins interact with the TAD of RelA(p65) [116]. TAF proteins are components of TFIID, as well as other co-activator complexes, like SAGA, STAGA and TFTC [136-139]. These complexes normally contain HAT activity and are involved in transcription regulation of large groups of genes, so are sometimes considered GTFs. TAFs and positive cofactor 4 (PC4) were shown *in vitro* to be necessary for RelA(p65) directed transcription and TAF1 ($TAF_{II}250$), TAF6 ($TAF_{II}80$) and TAF11 ($TAF_{II}28$) were found to bind directly to the TAD of RelA(p65) [117]. TAF1 is a large protein and has many different functions (reviewed in [140]). It is a member of many complexes, and stabilises these complexes by making contacts with other TAFs within the same complex. TAF1 can interact with many TFs, and also binds directly to initiator elements. It has acetylation, kinase and ubiquitin ligase activity, and can modify histones as well as GTFs. TAF6 binds to DPE in core promoters, is related to H4 and can bind DNA with a histone fold like domain [141]. TAF11 interacts with TFIIA at core promoters, so is likely to be important for PIC formation [142].

2.6.2 Mediator

Mediator is a large multi-protein complex containing around 30 proteins [143]. It was initially purified in many ways by different groups and therefore is known by many different names, although a unified nomenclature is now in use [144]. Most of the Mediator proteins are conserved between yeast and human, indicating the crucial role of the Mediator complex in transcription [145]. The Mediator complex interacts with RNAPII to form a holoenzyme [146], which is often the form of RNAPII recruited to the promoter region upon transcription activation. Two structurally distinct forms of Mediator have been shown, one with RNAPII and one without, indicating that a structural change could occur in the Mediator upon binding to RNAPII [147]. In yeast, a core Mediator subunit Med17 (Srb4) is as crucial for RNAPII transcription as the largest subunit of RNAPII itself, implying that Mediator has a role in regulating

transcription of nearly all genes [148]. Various subunits are responsible for binding to different TFs (listed in [149]), and in the case of the Nuclear hormone receptors (NHRs) the interaction is often ligand dependent. It is thought that through the interaction of TFs, the Mediator can coordinate many different signals, to regulate transcription by RNAPII appropriately. Mediator is known to interact with RelA(p65) via the TAD [120]. It was also shown to be important for NF-κB dependent reporter expression *in vitro* [150]. However, at one promoter (A20) Mediator was detected to be associated even before TNFα stimulation in Jurkat cells [151].

2.6.3 PARP-1

Poly ADP-ribosylpolymerase 1 (PARP-1) catalyses the transfer of ADP-ribose from NAD^+ to nuclear proteins, including chromatin proteins, to form long, branched polymers (reviewed in [152]). A role for PARP-1 in transcription was first indicated with the identification that PARP-1 was the main active component of TFIIC, which is required for accurate initiation of RNAPII transcription under some circumstances [153, 154]. PARP-1 has been found to activate NF-κB dependent reporter expression in transient transfection experiments independent of its enzymatic and DNA binding activity [155, 156]. PARP-1 activation of NF-κB is synergistic with p300 [157], which can acetylate PARP-1 to increase its interaction with p50 [158]. The acetylatable residues of PARP-1 were found to be important for Mediator and p300 activation of NF-κB dependent transcription [158].

2.6.4 p300/CBP

The homologous proteins p300 and CBP are large multi-domain proteins that can bind directly to many TFs and activate transcription of a wide variety of genes (reviewed in [159]). They regulate transcription by acting as a bridge between TFs and GTFs (TBP, TFIIB, TFIIE and TFIIF) (reviewed in [160]) and other cofactors (e.g. P/CAF [161]) and also by use of their acetyltransferase activity (reviewed in [162]). p300/CBP interacts with many proteins and is a component of enhanceosomes [163]. p300/CBP can acetylate core histones and non-histone proteins *in vitro*, providing a model whereby these HAT cofactors activate transcription by modifying nucleosomes and TFs in the cell upon activation (reviewed in [159, 164]). *In vitro* transcription experiments revealed that acetyl Coenzyme A (coA) [165] and histone

tails [166] are necessary for p300 activated transcription from promoters on DNA templates reconstituted with regularly spaced nucleosomes ("chromatin" templates).

p300 and CBP were found to activate NF-κB reporter gene expression [167] and to bind the TAD of RelA(p65) [115]. The TAD interaction with CBP was found to be independent on phosphorylation, while a RHD interaction was dependent on PKA phosphorylation of serine 276 [99]. Activation of RelA(p65) by p300/CBP acetyltransferase activity is also due to direct lysine acetylation of RelA(p65) (as well as acetylation of the histones) [168, 169]. The precise mechanism of how p300/CBP activates transcription is investigated using *in vitro* transcription experiments and is thought to be similar for all TFs regulated by p300. p300 activated transcription when added before PIC formation indicating that it functions by providing an effective transcriptional template for productive PIC formation [170]. It also has been shown to activate transcription synergistically with the transcription elongation factor, TFIIS [171], indicating it can also activate transcription by enhancing effective elongation by RNAPII.

There is evidence that p300 can also repress transcription *in vitro* using "chromatin" templates made with purified factors, and transcription reactions using purified RNAPII and GTFs [172]. In this system, p300 did not effect transcription of activated transcription with or without acetyl CoA on *naked* templates. However, on "chromatin" templates p300 repressed activated transcription when added without acetyl CoA and did not effect the transcription with acetyl CoA. By altering the time of addition of p300 the authors show p300 was specifically inhibiting a stage of transcription before initiation of the first phosphodiester bond, and could prevent initiation despite the presence of a pre-assembled initiation complex. In this system, neither p300 nor steroid receptor co-activator 1 (SRC-1) could activate transcription further, indicating that the purified system did not recapitulate a fully functional transcription assay. However, in a system for assaying productive transcription elongation, a similar effect was seen, indicating the possibility that acetyl CoA levels in the cell might regulate the transcriptional activation or repression by p300 [171].

2.6.5 P/CAF

P/CAF is a HAT protein, homologous to GCN5 and a member of various large complexes that acetylate histones as well as non-histone proteins. P/CAF has been

shown to be an NF-κB co-activator [173], and also to acetylate RelA on specific lysines to regulate the duration of transactivation [169].

2.6.6 p160/SRC family

Three homologous HAT proteins make up this family called steroid receptor co-activator 1, 2 and 3 (SRC-1, SRC-2 and SRC-3). There are many alternative names: SRC-1 = NcoA-1; SRC-2 = NcoA-2, GRIP1, TIF2 and SRC-3 = NcoA-3, ACTR, TRAM1, AIB1, RAC3, P/CIP. They were first identified as factors interacting ligand dependently with nuclear hormone receptors to activate transcription (reviewed in [174]). They are found in large HAT complexes, which can activate transcription driven from many different TFs (reviewed in [175]). All three proteins have also been shown to have a role in NF-κB transcriptional regulation. The role for SRC-1 was shown by injection of anti-SRC-1, which led to inhibition of NF-κB reporter expression [173]. This was relieved upon overexpression of SRC-1 but not -2 or -3. ASC2, a protein upregulated in some cancers is a component of specific SRC-1 and p300/CBP containing complex (distinct from the standard SRC-1 complex) [176]. ASC2 also interacted with p50 and RelA(p65) and activated reporter genes in transient transfection experiments [177]. SRC-2 and SRC-3 were also reported as NF-κB co-activators [173, 178]. SRC-3 is present in the cytoplasm of cells grown in serum free media, and in the nucleus of cells stimulated with TNFα or insulin [179], probably due to phosphorylation of SRC-3 by the IKK complex [180].

ChIP experiments in 293T cells investigating transcriptional cofactor recruitment to the NF-κB regulated IκBα promoter showed differences in the SRC recruitment. SRC-2 was found to be present at the promoter even before TNFα stimulation [181]. Upon TNFα stimulation, SRC-1 associated with the promoter while SRC-2 dissociated and left the nucleus. SRC-3 associated quickly with the promoter upon TNFα treatment, then dissociated and re-associated later. This indicates that the function of each SRC protein in the regulation of the IκBα gene may be different.

2.6.7 PRMT1 and 4

Protein arginine methyl-transferases (PRMTs) are transcriptional co-activators that methylate histone and non-histone proteins. PRMT4 (also called CARM1) was shown

to activate a subset of NF-κB dependent genes *in vivo* [182]. It interacts with the RHD of RelA(p65), and forms a complex with p300 and NF-κB in nuclear extracts [182]. Unpublished data from our group also indicates PRMT1 as a co-activator of NF-κB (Covic and Hottiger, unpublished).

2.6.8 Brg1

Interestingly there are very few reports demonstrating that ATP dependent remodellers are required or recruited by RelA(p65) for transcription regulation. Indeed, *in vitro*, Brg1 had no effect on human immunodeficiency virus 1 - long terminal repeat (HIV1-LTR) transcription controlled by RelA(p65) [183]. However, Brg1 could be involved in transcription of specific NF-κB genes since Brg1 was shown to be recruited to the IL-8 gene upon IL-1β stimulation [184]. Also reporter assays using the major histocompatibility class 1 promoter indicated that Brg1 and P/CAF synergistically activated NF-κB dependent reporter expression [185]. Additionally Brg1 was recruited to oligonucleotides containing NF-κB binding sites in the granulocyte, macrophage colony stimulating factor (GM-CSF) promoter [186]. Brg1 was also shown to be important for IFNγ enhanceosome activity, indicating that chromatin remodellers may be important for transcription of certain genes, including ones regulated by NF-κB [187].

2.6.9 P-TEFb

The positive elongation factor b (P-TEFb) consists of cyclin T1 and Cdk9 (reviewed in [12]). It stimulates transcription elongation by phosphorylation of the RNAPII CTD. Cdk9 was shown to activate HIV1-LTR (contains two NF-κB binding sites) driven reporter expression, whereas Cdk9 kinase deficient mutant repressed reporter expression [188]. RelA(p65) was found to interact with cyclin T1 from cellular extracts and P-TEFb was recruited to IL-8 promoter upon TNFα stimulation. This coincided with RNAPII recruitment and phosphorylation at serine 2 and 5 of the CTD. Therefore, P-TEFb is an NF-κB cofactor that can regulate transcription at a stage after PIC formation, during the elongation of RNAPII.

2.6.10 HDACs

HDAC containing co-repressor complexes contain the homologous proteins N-CoR and SMRT. These proteins interact directly with unliganded nuclear hormone receptors and other TFs to repress their activity (reviewed in [78]). HDACs de-acetylate histones as well as non-histones to regulate transcription. HDACs are also members of other complexes, that often contain chromatin remodelling factors (reviewed in [78]). HDACs fall into three classes by sequence homology, class 1 members are Rpd3 (yeast), HDAC1, 2, 3, and 8 (human), class 2 members are Hda1 (yeast), HDAC 4, 5, 6, 7, 9 and 10 (human), class 3 members are Sir2 (yeast) and the SIRT proteins (1 to 7 in humans). Micro-array analysis of bladder or breast cancer cells treated with various HDAC inhibitors, identified that as many genes were upregulated as downregulated upon treatment implying that HDAC activity is not always involved in transcriptional repression [189].

There is evidence that N-CoR, SMRT and HDAC1, 2 and 3 are involved in regulation of NF-κB. SMRT was shown to repress NF-κB dependent expression of an IL-2 reporter, and this was relieved upon treatment with the HDAC inhibitor, trichostatin A (TSA) [190]. In a different report, HDAC 1 and 2 as well as N-CoR (but not SMRT) were found to repress TNFα stimulation of NF-κB dependent reporter expression in NIH3T3 cells [191]. Treatment with TSA led to activation of IL-8 (an NF-κB regulated gene) and coincided with increased acetylation of the promoter region. The HDACs were suggested to be recruited to NF-κB dependent promoters and this could be through direct interaction of HDAC 1 with the RHD of RelA(p65) [191]. However, *in vitro* transcription reactions using a "chromatinised" HIV1-LTR template activated by RelA(p65), showed that repression by HDAC1 required Gal4 sites and Gal4-HDAC1 [192]. This indicates that HDAC1 recruitment by HDAC1/RelA(p65) interaction may not be general and could be gene specific. Alternatively a whole repressor complex could be recruited by multiple protein/RelA(p65) interactions. The tumour suppressor protein ARF was shown to inhibit NF-κB by inducing association between RelA(p65) and HDAC1 from extracts [135]. This was dependent on the ATR kinase and on threonine 505 of RelA(p65).

HDAC3 (not HDAC 1 or 2) was found to repress NF-κB synthetic reporter expression in 293T cells [193]. HDAC3 (not 1) was also shown to interact with RelA(p65) by co-immunoprecipitation studies [193]. One difference to the report

described above where HDAC1 and 2 repressed NF-κB dependent expression, and HDAC1 associated with RelA(p65) was the different cell types used. Over-expression of HDAC3 led to less acetylated RelA(p65), relocation of RelA(p65) to the cytoplasm (dependent on IκBα) and increased interaction with IκBα in the presence of TNFα in 293T cells [193]. This supports a model whereby de-acetylation of nuclear RelA(p65) is involved in transcription termination by RelA(p65) association with IκBα and relocation to the cytoplasm. Another report confirmed the interaction between HDAC3 and RelA(p65) *in vitro* and showed that HDAC3 could directly de-acetylate RelA(p65) [169]. After cells were stimulated by attachment to laminin, RelA(p65) and co-activators were recruited to the IL-8 and cIAP-2 promoters, whilst HDAC3 was transiently released from the promoters, and this release was dependent on IKKα [194]. IKKα was found to phosphorylate SMRT, and the phosphorylated innactive form was bound to the promoter region, with the same kinetics as the co-activators [119]. IKKα phosphorylated SMRT could not interact with RelA(p65) *in vitro*, resulting in derepression of NF-κB [119]. This is an example of a switch from a co-repressor bound RelA(p65) containing promoter/enhancer complex to a co-activator containing complex.

ChIP experiments investigating transcriptional cofactor recruitment to the NF-κB regulated IκBα promoter showed differences in the HDAC protein recruitment and therefore in their functions [181]. HDAC1 protein was found to be present at the promoter in 293 cells before stimulation, but surprisingly for a co-repressor its presence was found to be enhanced at 30 min and 120 min after THFα stimulation, showing some oscillation (the same pattern as for SRC-3). In contrast, there were no obvious differences in the HDAC2 and HDAC3 levels found at the IκBα promoter. However, HDAC2 did not regulate IκBα reporter gene expression when over-expressed or when knocked down, whereas HDAC3 was found to be a classical co-repressor in these experiments. SMRT association at 30 min was stimulated by TNFα, while NcoR associated at 60 minutes [181]. Together these studies indicate that the repression of NF-κB is mediated through many different HDAC containing co-repressor complexes. However, the components of the complexes, order of recruitment and targets are probably gene, stimuli and cell type specific.

2.6.11 SIRTs

SIRT proteins are class 3 HDACs that are NAD^+ dependent for HDAC activity (reviewed in [195]). SIRT1 has been found to de-acetylate histones and non-histone proteins. SIRT1 interacts with RelA(p65) and de-acetylates lysine 310 [196]. SIRT1 was shown by ChIP to bind to the promoter regions of some genes (e.g. cIAP-2) but not others (e.g. IκBα). Upon TNFα treatment, SIRT1 was released from the cIAP-2 promoter, whilst RelA(p65), p50, RNAPII were recruited. Acetylation of H3K14 was also increased. In the presence of Resveratol (an agonist of SIRT1) and TNFα, recruitment of the activators of transcription was delayed whilst SIRT1 remained associated [196].

2.6.12 IκBα in the nucleus

Expression of IκBα is under the control of NF-κB and upon IκBα synthesis, it can enter the nucleus, and bind to promoter/enhancer bound NF-κB resulting in NF-κB removal from the chromatin, and translocation to the cytoplasm [197]. The expression of the other IκB proteins are not controlled by NF-κB, therefore only IκBα is involved in termination of the NF-κB response, and allows transient activation of gene expression.

2.6.13 Other proteins

Many other proteins have been shown to activate or repress NF-κB dependent transcription in transient transfection reporter assays, without affecting IκB degradation or RelA(p65) nuclear import. For example, E1A 13S (adenovirus early region 1A) [198], TLS/FUS [199], 53BP2 (p53 binding protein 2) [200] and BRCA1 (breast cancer 1) [201] activate NF-κB dependent transcription, while papilloma virus proteins E6 [202], c-Myc [203], HSCO (over-expressed in hepatocellular carcinomas) [204], SINK (p65-interacting inhibitor of NF-κB) [205]. Epstein-Barr virus BZLF-1 (a basic leucine zipper DNA binding protein)[206] repress.

2.7 Enhanceosomes

Enhanceosome is the term coined to describe the complex composed of trans-activating factors and chromatin components that integrate signals to precisely regulate transcription of a particular gene, or set of genes. One of the most well

studied enhanceosomes contains RelA(p65) and the interferon beta (IFN-β) enhancer and illustrates the key functions of enhanceosomes to temporally, specifically and combinatorially control gene expression [163, 207]. The IFN-β gene is activated by virus infection, by induction of NF-κB, interferon regulatory factors (IRFs) and ATF-2/c-Jun heterodimer. Together with HMG I(Y), these transcription factors cooperatively bind the cognate DNA sequences resulting in transcriptional synergy. After the initial binding and looping of DNA to accommodate the binding, enhanceosome assembly is dynamic and cofactors are sequentially included into the enhanceosome at precise times after virus infection. Early in cofactor recruitment, the enhanceosome recruits non-phosphorylated RNAPII, which is competent for transcription initiation [207]. The histone acetyl transferase, GCN5, is recruited and acetylates histones and HMG I(Y), followed by hSWI/SNF mediated chromatin remodelling. The resulting chromatin is able to bind to TFIID, and TBP binding to the TATA box results in the shifting of a positioned nucleosome away from the transcription start to 36 nucleotides downstream [187]. From these events it follows that the IFN-β enhanceosome activates transcription by increasing the rate of PIC formation [207]. The position of the nucleosome is crucial in enhanceosome function, as synthetically positioning the nucleosome to the downstream site led to the loss of temporal control of IFNβ expression, as well as loss of signal specificity [208]. The series of acetylation and deacetylation events described for the activation of IFNβ gene transcription by the enhanceosome is complex. Recently is was shown that HDAC6 activates enhanceosome transcription initiation through IRF3, while HDAC1 and 8 repressed the transcription [209].

3 Cell type specific regulation of NF-κB

3.1 Cell types and Cell differentiation

In our bodies we have many different tissues and cells, all of which perform a specific function due to the presence of specialised cells. Most of the cells are terminally differentiated and in order to renew these cells, a small number of stem cells have the ability to replicate, and to differentiate into a number of specific types of cell. Differentiated cells have a specific morphology and physiology resulting from a precise pattern of gene expression. This pattern is achieved during differentiation by genomic imprinting (e.g. DNA methylation patterns), chromatin remodelling events, heterochromatin formation and special nuclear structures (reviewed in [26]).

3.2 Accessibility to gene control regions

One of the major ways of regulating transcription of specific genes (including NF-κB dependent genes) in different cell types is by regulating the accessibility of the gene control regions (reviewed in [73]). This can be through cell type specific patterns of nuclear structures, like chromosome territories and heterochromatin, as well as local chromatin structures. All of these levels of chromatin structure are interdependent. For example, the cell type specific positioning of a chromosome within the nucleus will affect its heterochromatin status.

3.2.1 Local chromatin structure

Different cell types have different patterns of histone modifications and positioned nucleosomes in the region of specific genes. An example where the local chromatin structure directly regulates the transcriptional potential of RelA(p65) is the chromatin structure surrounding the E-selectin promoter [210]. E-selectin is an adhesion protein expressed only in endothelial cells and is involved in the binding of leukoctyes at sites of infection and inflammation. Expression of E-selectin has been found to be up-regulated by inflammatory cytokines (like TNFα) and is regulated by NF-κB, ATF-2/c-Jun and HMGI(Y). When the proximal promoter of E-selectin was used in reporter assays, it was activated in non-endothelial cells, indicating the endogenous E-selectin expression is regulated by its endogenous chromatin status. The E-selectin

promoter region was found to be more accessible to micorcoccal nuclease treatments in HUVEC (human umbilical vein endothelial cells) than in UASMC (umbilical artery smooth muscle cells) [210]. This indicates that this particular gene control region is more accessible to protein factors in endothelial cells than in the other cell type. How this is achieved is not known, although it was shown to probably be independent of H3K9 methylation. Upon TNFα stimulation there was no change in the micrococcal nuclease accessibility of the E-selectin promoter in UASMC cells, whereas the promoter region in HUVEC cells became more accessible and transcription factors and co-activators were recruited [210].

3.2.2 Heterochromatin and higher order chromatin structure

Electron microscopic studies show that the nucleus of stem cells generally have very little heterochromatin "keeping the options open" whereas once the cell is committed to a specific differentiated cell type it has a specific pattern of heterochromatin (reviewed in [62]). Analysis of various types of mouse cells, indicated that the positioning of heterochromatin is cell type specific [211]. This allows the repression of a vast number of genes, whose protein products are not necessary for the function of the specific differentiated cell. Other aspects of chromatin structure are also cell type specific, although the mechanism of higher order chromatin structure regulation in different cell types is mostly unknown. However, special AT rich binding protein 1 (SATB1) is an example of a cell type specific protein that can regulate higher order chromatin structure, nuclear architecture and gene expression [212]. SATB1 binds to DNA at base unpairing regions (BURs), which are found at many places in the genome. The BURs surrounding the immunoglobulin heavy chain intronic enhancer (contains an NF-κB site) are necessary for tissue specific transcription (in B cells) [213]. SATB1 is expressed mainly in T cells, and binds to the bases of looped out chromatin [214]. It is thought to regulate chromatin structure and transcription by recruitment of chromatin remodeling complexes [212] and establishment of specific histone modification patterns over SATB1 controlled regions [215]. Cell type specific heterochromatin and chromatin structure are likely to have a large role in the regulation of NF-κB dependent transcription. However, the effect of higher order chromatin structure on cell type specific, NF-κB dependent transcription has not directly been studied.

3.2.3 Chromatin territories and the nuclear periphery

Cell specific alteration of the position of a gene within the nucleus could regulate the potential transcriptional activity of this gene. Genes situated on the outside of a chromatin territory (looped into the interchromatin compartment) are usually more active than those within the chromatin territory or at the nuclear periphery (Section 1.2.8) (reviewed in [26]). For example, fluorescence in-situ hybridisation analysis showed that the IgH and κ light chain loci (both regulated by NF-κB) are localised to the nuclear periphery in non-B cells, whereas they are localised internally in chromatin territories in pro-B cells (immature B cells) [66]. A model is proposed that for IgH and Igκ genes, transcription and recombination at these loci is restricted in the default state by association with the nuclear periphery. Looping out of gene regions from the chromatin territories into the interchromatin space can activate the gene locus in a specific cell type. For example, the IgH locus is looped from the chromatin territory in developing B cells, bringing together V and H regions, facilitating recombination (reviewed in [216]). In non-B cells like pro-T cells there is only limited expression of some germline V transcripts from the IgH loci, while in pro-B cells the V array of transcripts is transcribed, and this allows VDJ recombination to occur. Ultimately the immunoglobulin locus positioning allows NF-κB dependent transcription of germline transcripts during class switching as well as immunoglobulins in stimulated mature B cells (reviewed in [26] and [216]).

3.3 Receptors/signalling cascades

Different cell lines express different cell surface receptors and signal transduction molecules. This means that each cell is responsive to a particular pattern of stimuli. For example, LPS is a NF-κB activating compound found on the outer membrane of gram-negative bacteria (reviewed in [217]). LPS binds to CD14 cell surface protein expressed on monocytes, monocyte derived macrophages and other myeloid cells. LPS binding to CD14 results in the activation of Tol like receptor 4 (TLR4) and the activation of various signalling pathways and TFs that regulate the expression of many different inflammatory cytokines. Since CD14 and TLR4 are only expressed on the surface of myeloid cells, LPS can activate NF-κB only in these cells. In a similar way, only B cells express B-cell receptors, and only T cells express T-cell receptors, both of which signal to NF-κB to activate a specific set of genes.

Other proteins that contribute to NF-κB signalling are also expressed in certain cell types. One of the major upstream activators of NF-κB is the PKC family of kinases. They are differentially expressed in different cell types, and activated by different stimuli, resulting in the activation of many different signalling cascades including IKK phosphorylation and ultimately NF-κB activation [218, Castrillo, 2001 #251].

3.4 Expression pattern of NF-κB family members

Different cell types have specific expression patterns of the NF-κB proteins. The major NF-κB dimer consists of RelA(p65) and p50 and both are widely expressed. RelB is expressed in parts of the thymus, lymph nodes and Peyers patches while c-Rel is expressed in haematopoietic and lymphocyte cells (reviewed in [219]). This limits the possible pattern of formation of NF-κB dimers in different cell types. The different functions of the NF-κB proteins in different cell types can be seen by the differences in phenotype of the knock out mice. RelA(p65) -/- mice die as embryos at gestation day 15-16 [220]. However, the other NF-κB knock out mice do not have developmental defects, but they do show defects in immune responses (reviewed in [83]). For example, NF-κB1 -/- and NF-κB2 -/- mice develop normally, but have deficient adaptive immune responses; in particular, they cannot activate lymphocytes. RelB -/- mice also have defective adaptive immunity and dendritic cell development, and die on birth due to organ inflammation. c-Rel -/- mice also have adaptive immunity defects as well as macrophage defects (reviewed in [83]).

Electromobility gel shift assays (EMSA) have shown that the DNA binding dimers that form in each tissue changes during development [221]. In mature B cells, the most prominent NF-κB dimer is the heterodimer c-Rel/p50 [222] [223]. By contrast, stimulation of the pre-B cells 70Z/3 (κ light chain locus is rearranged but not transcriptionally active) led to the activation of RelA(p65)/p50 dimers. Upon B cell maturation c-Rel expression was found to increase, and the predominant active NF-κB in the mature B cell line WEHI 231 was found to be the constitutive nuclear c-Rel/p50 [222]. The reason for the constitutive nuclear localization of c-Rel/p50 in mature B cells is not fully understood. The expression pattern and DNA binding activity of nuclear NF-κB was also analysed in monocytes, monocyte derived macrophages and dendritic cells [224]. p50 homodimers were detected in all three cell

types in non-stimulated conditions. Upon LPS stimulation RelA(p65)/p50 and RelB/p50 shifts were up-regulated in macrophages and dendritic cells. The RelB/p50 gel shift was extremely increased in LPS treated dendritic cells compared to the other cell lines [224]. In fibroblasts the majority of TNFα regulated NF-κB genes are transcribed by RelA(p65)/p50 heterodimers, since TNFα treated NF-κB1-/- RelA-/- fibroblasts showed no NF-κB gel shift, no transcription of various κB reporters and very little NF-κB responsive endogenous mRNA was detected [96]. Each dimer is likely to activate a different, but overlapping set of genes since there is some functional redundancy of these proteins as transcription factors [96].

The tissue specificity of NF-κB protein expression is further complicated by the inducible expression of many NF-κB proteins. For example, expression of RelB, c-Rel, p105, as well as IκBα are regulated by NF-κB itself. Therefore, the availability of active NF-κB proteins is determined by the cell type, as well as cell status and leads to a specific pattern of NF-κB dimers.

3.5 Cofactors of transcription

Transcription of genes is coordinated by the action of transcription factors and cofactors (Section 1.3). If these proteins are expressed only in a subset of cell types, then they will only be able to regulate transcription in a subset of cell types. For example, the protein Ikaros was found to be a lymphocyte transcriptional repressor that can bind to control regions of terminal deoxynucleotidyltransferase and other lymphoid regulated genes to recruit them to heterochromatin [225].

There is so far only one known cell type specific cofactor of NF-κB dependent transcription, which is TAF4b (also called $TAF_{II}105$ [226]), a TAF4 ($TAF_{II}130$) homologue. It is expressed in many cell types, but it is probably part of TFIID only in B cells and mouse gonads [227]. $TAF_{II}105$ was found to interact with the transcription activation domain of RelA(p65) and to enhance transcription of reporter genes [118]. Dominant negative $TAF_{II}105$ sensitized 293T cells to TNFα induced apoptosis indicating $TAF_{II}105$ might normally be involved in the transcription activation of anti-apoptotic genes. Accordingly anti-apoptotic A20 gene was upregulated by TNFα treatment in normal cells, but not in cells expressing dominant negative $TAF_{II}105$. Also A20 expression rescued the TNFα sensitivity of $TAF_{II}105$ dominant negative expressing phenotype [228]. In agreement with $TAF_{II}105$ having a

specific role in activating NF-κB dependent anti-apoptotic genes, transgenic mice with the dominant negative $TAF_{II}105$ show lymphocyte apoptosis and have lower levels of A20 [229]. The dominant negative $TAF_{II}105$ mice also produced lower levels of antigen specific antibodies. The mode of transcriptional activation by $TAF_{II}105$ could be by bridging the interaction between NF-κB and the GTFs.

TAF $_{II}$105 knock out mice are viable and were not detected to have a major defect in development or metabolism [230]. A defect in B cells was not detected in the knock out mice, indicating that other TAFs might be able to carry out some of the processes normally done by TAF $_{II}$105 [230]. However, knock out female mice were infertile, despite being able to mate, ovulate and carry surrogate pregnancies. The ovaries of the knock out mice were half the weight of wild type counterparts indicating that mice lacking $TAF_{II}105$ had oocyte developmental defects. Micro-array analysis indicated that 1% of ovarian genes in knock out cells were down regulated 2 fold or more, and some of these are known to be involved in oocyte development. Of these genes, there are at least 2 (cyclin D2 [231] and aromatase [232]) that are regulated by NF-κB. However, it is likely that $TAF_{II}105$ exerts important effects through other transcription factors, like c-Jun [233].

4 Aim of thesis

NF-κB is an important transcription factor for the regulation of over 300 genes. Precise control of the pattern of NF-κB dependent gene expression in each cell type is required for the coordinated reaction of each cell in an organism. Cell type specific regulation of NF-κB gene expression can be regulated in many ways. One way would be through the use of cell type specific transcription cofactors, although so far only one has been detected. Therefore, the aim of this thesis was to identify other cell-type specific cofactors of NF-κB, in particular RelA(p65), and to investigate their effect on NF-κB dependent gene expression.

Results

5 Research Articles

5.1 Identification of Novel and Cell Type Enriched Cofactors of the Transcription Activation domain of RelA (p65 NF-κB).

5.2 MYBBP1a is a novel co-repressor of NF-κB.

5.3 Modulatation of gene-expression by p300-mediated acetylation of RelA/p65.

My contribution to this paper was the *in vitro* transcription experiments on chromatinised templates, and the interaction studies between p300 and GST-RelA/p65 wild type or KTR mutant.

5.4 Acetylation of Poly(ADP-ribose) Polymerase-1 by p300/CREB-binding protein regulates coactivation of NF-κB-dependent transcription.

My contribution to this paper was the initial experiments identifying Mediator interactions with PARP-1 and in particular the Med14 (DRIP150) as an interaction partner for PARP-1.

research articles Journal of proteome research

Identification of Novel and Cell Type Enriched Cofactors of the Transcription Activation Domain of RelA (p65 NF-κB)

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RelA (NF-κB) is a transcription factor inducible by distinct stimuli in many different cell types. To find new cell type specific cofactors of NF-κB dependent transcription, we isolated RelA transcription activation domain binding proteins from the nuclear extracts of three different cell types. Analysis by electrophoresis and liquid chromatography tandem mass spectrometry identified several novel putative molecular partners. Some were strongly enriched in the complex formed from the nuclear extracts of specific cell types.

Keywords: NF-κB • RelA • p65 • transcription • cofactors • mass spectrometry

Introduction

The mammalian NF-κB family of inducible transcription factors is responsible for regulating specific sets of genes in many cell types (reviewed in ref 1). There are 7 proteins within this family (RelA/p65, c-rel, RelB, p105, p50, p100, p52) which can form combinations of homo- and heterodimers. NF-κB responsive elements are found in the enhancer and proximal promoter regions of a wide variety of genes; notable examples are immunoglobin genes and the long terminal repeat of the HIV-1 genome. Misregulation of NF-κB is linked to a wide variety of human diseases, for example various lymphomas, myelomas, leukaemia's, carcinomas and adenocarcinomas (reviewed in ref 2). The critical role of NF-κB, in particular the RelA/p65 transcription factor, in maintaining normal cellular events is apparent in RelA knockout mice that die at E15.5-E16.5 and show TNF-dependent liver apoptosis and defects in active lymphocyte development.[3]

RelA contains a bipartite carboxyl terminal acidic transactivation domain (TA). When fused to the Gal4 DNA binding domain, the TA of RelA activates expression of Gal4 dependent reporter genes.[4] NMR and circular dichroism experiments indicate that the TA has no stable secondary structure, although the TA could form an alpha-helix when in an hydrophobic solvent implying that the TA may form a stable structure when part of a higher order complex.[4] Full transcriptional activity of RelA-TA is dependent on repeats within the conditional alpha-helix, which is modeled to be an amphipathic helix.[5] The TA has subsequently been shown to regulate transcription by interaction with transcription cofactors, which can have a positive (coactivators) or negative (corepressors) effect upon inducible transcription.

Transcriptional coactivators reported to be involved in the regulation of NF-κB dependent genes are CBP/p300,[6,7] SRC-1/NcoA-1,[8] P/CAF,[9] TBP, $TAF_{II}250$, $TAF_{II}80$, and $TAF_{II}28$, TFIIB,[10] $TAF_{II}105$[11,12] Mediator,[13] PC4,[13] E1A 13S,[14,15] TLS/FUS,[16] RAC3,[17] 53BP2[18] and BRCA-1,[19] RNA helicase A,[20] PRMT4,[21] and PARP1.[22] There are also many transcriptional corepressors that have been shown to mediate repression of NF-κB dependent gene expression, such as HDAC1 and 2,[23] HDAC3,[24] HSCO,[25] SINC,[26] and ASC-2.[27] Many of these cofactors are known to interact with the transcription activation domain of RelA (CBP, p300, Mediator, TBP, the TAFs, and TFIIB). Only one cell type specific cofactor of NF-κB is known, $TAF_{II}105$. This B-cell enriched coactivator is a component of TFIID and can bind directly to the RelA-TA.[11,12] Interestingly, it is required for expression of a subset of NF-κB dependent genes, including antiapoptopic A20.[28]

In this work, we attempt to identify new cofactors of RelA that interact with the transcription activation domain of RelA, and in particular, cell type enriched or specific cofactors. We isolate proteins that interact with the transcription activation domain of RelA (RelA-TA) from the nuclear extract of 3 different human cell types by affinity purification. The cell types used were Jurkat cells (acute T cell leukaemia), THP1 cells (acute monocytic leukaemia) and HeLa-S3 (cervix epithelial adenocarcinoma). These cell lines are responsive to specific sets of NF-κB stimuli and therefore could contain different sets of transcriptional cofactors involved in NF-κB dependent transcription. We identified the high molecular weight (over 100 kDa) interaction partners of RelA-TA by electrospray mass spectrometry after electrophoretic separation. This required adjustment and optimization of the standard purification procedures to yield a protocol that we find to be very reproducible. We confirm that CBP, p300, and subunits of the

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Mediator complex are RelA-TA binding proteins and extend the knowledge by analyzing the interaction of RelA-TA with specific in vitro translated subunits of the Mediator complex. We find Med14 (drip150, trap170), Med17 (drip77, trap80) and Med24 (drip100, trap100) can interact weakly with the RelA-TA. We have found novel RelA-TA interacting proteins such as the transcriptional regulators TRRAP and MYBBP1a. Other proteins with the potential to bind to the RelA-TA are the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), ATR, IQGAP3, DRIM, hGCN1L1 as well as some uncharacterized hypothetical proteins. We also found some cell type enriched RelA-TA binding proteins such as MYBBP1a and DRIM (from Jurkat nuclear extracts), IQGAP3 (from THP-1), and hGCN1L1 (from HeLa). Our results indicate that these proteins, most of which are hypothetical or poorly characterized, could have a role in transcription regulation in different cell types.

Experimental Section

Apparatus. A Typhoon 9400 fluorescence scanner was used for gel analysis. A SCIEX QSTAR Pulsar i (Concord, Ontario, Canada) hybrid quadrupole-time-of-flight instrument equipped with a nanoelectrospray source and interfaced to an LC−Packings Ultimate (Amsterdam, Holland) HPLC system was used for all mass spectrometry measurements.

Procedures. Cell Culture Technique and Nuclear Extract Preparation. Nuclear extracts were prepared from suspension cells cultured by standard sterile techniques in RPMI (Gibco), 10% FCS, plus penicillin and streptomycin. 1−2 L of cells were harvested when growing exponentially at a density of 0.5−1 × 10^6 cells/ml and cell pellets were washed in PBS. All the following steps were performed at 4 °C. The cell pellet was resuspended in buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.2 mM PMSF, 1 μg/mL pepstatin, bestatin, and leupeptin), transferred to multiple 2 mL tubes and incubated on ice for 5 min. Cells were centrifuged at 425 × *g* for 1 min, resuspended in 1 mL buffer A (per 2 mL tube) and centrifuged as above. Cells were resuspended in 3 pellet volumes (V) of buffer A* (buffer A plus 0.1% NP40), and centrifuged at 10 621 × *g* for 5 min when the cell cytoplasmic membrane was disrupted. This was between 3 and 5 min, as determined under the microscope by the movement of trypan blue dye into the cell. Supernatant (cytoplasmic extract) was discarded and the nuclei containing pellet washed in 1 mL buffer A, resuspended in 3 volumes buffer C (20 mM HEPES-KOH pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 1 mM DTT, 0.2 mM PMSF, 1 μg/mL pepstatin, bestatin, and leupeptin) and rolled for 25 min. This nuclear/membrane extract was centrifuged at 20 817 × *g* for 10 min, and the supernatant containing the nuclear extract was diluted (2×) in buffer D (20 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol) to adjust salt to 158 mM NaCl. The protein concentration in this nuclear extract was measured by a Bradford assay (BIO-RAD), flash frozen in liquid nitrogen, and stored at −80 °C for up to 1 year.

GST Pull Down Experiments with Nuclear Extracts. GST-RelA-TA (amino acids 431−551 AGE...ISS) was expressed in *E. coli* using standard protocols. The cells were lysed by passage through a French Press, and the lysate kept at −80 °C until use. Thawed lysates were diluted with ATP buffer (50 mM Tris-HCl pH 7.5, 2 mM ATP, 10 mM $MgCl_2$) for 10 min at 37 °C. Lysate/ATP buffer was diluted with binding buffer (20 mM HEPES-KOH pH 7.9, 0.1% NP40, 10% glycerol, 250 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 1μg/mL pepstatin, bestatin and leupeptin) with 20 μL/condition glutathione sepharose beads (Pharmacia) for 2−3 h 4 °C. Bound protein (approximately 10 μg/condition of GST-RelA-TA) was washed in wash buffer (20 mM HEPES-KOH pH 7.9, 0.1% NP40, 10% glycerol, 400 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 1 μg/mL pepstatin, bestatin and leupeptin) 3 times for 10 min each and in nuclear extract buffer (20 mM HEPES-KOH pH 7.9, 0.1% NP40, 10% glycerol, 158 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 1 μg/mL pepstatin, bestatin and leupeptin) 1 time. Nuclear extracts were thawed on ice, centrifuged 20 817 × *g* for 10 min and the protein concentration of the supernatant was measured. 4−10 mg of nuclear extract was incubated with glutathione beads/GST-RelA-TA for 4−6 h at a concentration of 0.5 mg/mL in nuclear extract buffer with (Figures 1 and 2) or without (data not shown) 50 μg/mL ethidium bromide. Beads/GST-RelA-TA/nuclear proteins were washed 3 times in wash buffer and 1 time in nuclear extract buffer. Proteins were eluted from beads by boiling in 4 μL of 10× SDS laemmli buffer, separated on a 3−8% gradient SDS-PAGE (Novex) and visualized by SyproRuby (Molecular Probes) according to the protocol supplied by the manufacturer. Gels were scanned using a Typhoon 9400 scanner (blue filter 488 nm). Image analysis and densitometry were performed with the software Quantity One (Bio-Rad), background levels were subtracted (50 cpm).

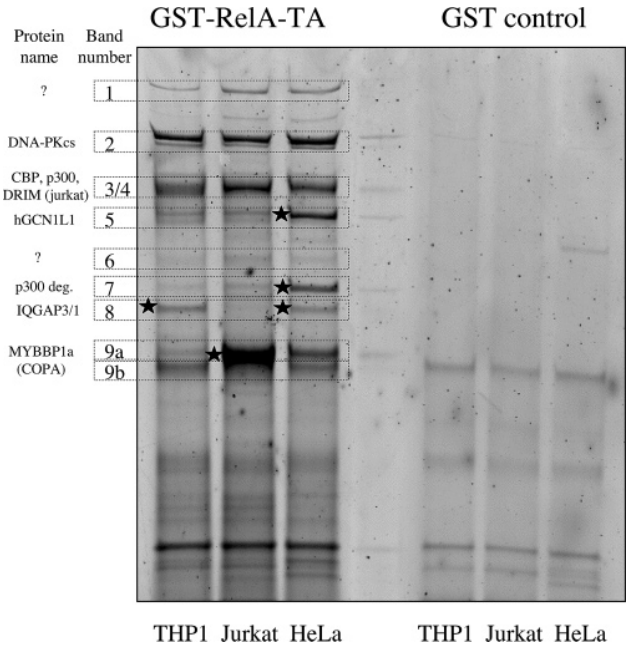

Figure 1. RelA-TA binds a different pattern of proteins from nuclear extracts of different cell types. GST-RelA-TA binding proteins from nuclear extracts were isolated, separated on a 3−8% gradient SDS-PAGE and stained with SyproRuby (Molecular Probes). The nuclear extracts were prepared from 3 different cell types as indicated (THP1, Jurkat and HeLa). Bands found in the GST-RelA-TA lane but not in the GST control lane are boxed, numbered and named. Bands that are found differentially between the cell types are labeled on the left side with a star. The proteins were identified by electrospray LC−MS/MS and properties of the proteins are given in Table 1.

Mass Spectrometry. Bands were excised from gels as cubes of 2.0 mm length. Proteins were manually in-gel digested with trypsin according to a described protocol.[29] Tryptic peptides were recovered in the supernatant of the digestion, concen-

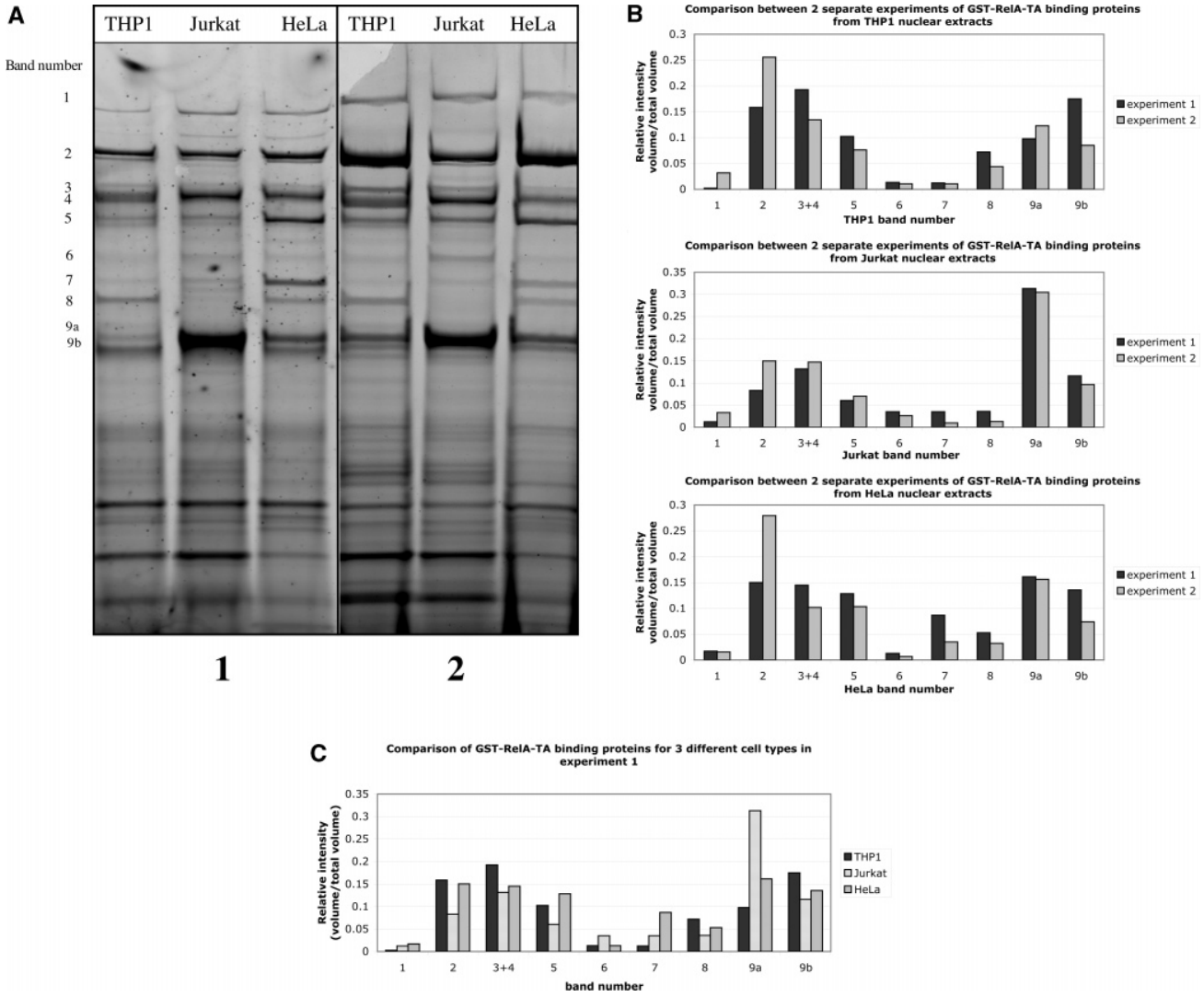

Figure 2. Results of the RelA-TA pull down experiments are reproducible. (A) The GST-RelA-TA interacting proteins from two separate experiments are shown. Bands were numbered as in Figure 1 and quantified. (B) Comparison of the intensity of GST-RelA-TA interacting protein bands from either THP1 (top), Jurkat (middle) or HeLa (bottom) nuclear extracts in two distinct experiments. The relative intensity of each band = intensity of band/intensity of whole lane. The relative intensity of each band from experiment 1 was compared to its counterpart in experiment 2. (C) Comparison of the intensity of GST-RelA-TA interacting proteins between the 3 different cell types (in experiment 1).

trated by evaporation to 15 μL and analyzed by liquid chromatography-tandem mass spectrometry (LC−MS/MS) on a SCIEX QSTAR Pulsar i (Concord, Ontario, Canada) hybrid quadrupole-time-of-flight instrument equipped with a nanoelectrospray source and interfaced to an LC−Packings Ultimate (Amsterdam, Holland) HPLC system. Separation was performed on a PepMap (LC−Packings, Amsterdam) reversed-phase capillary C18 (75 μm I.D. × 15 cm) column at a flow rate of 200 nL/min along a 52-min gradient of acetonitrile (0−40%). The Analyst instrument controlling software was used to perform peak detection and automatically selecting sequentially eluting peptides for collision-induced fragmentation (CID). The two most intense ions in the mass range 400−1200 with charge state +2 to +4 were selected for analysis after a 1 s survey scan. CID spectra were accumulated for 7 s for every precursor. Analyzed ions were excluded for 180 s from further analysis (the tolerance window for exclusion was 0.075 amu).

CID Data Generation. Collections of tandem mass spectra for database searching were generated from the Analyst files with the script Mascot.dll version 1.6b4 (Matrix Science, London). The tool was set to try to determine precursor charge state from the survey scan, after centroiding peaks at 50% height and merging datapoints within a distance of 0.1 amu. Charge state information thus determined was used whenever available, while all the default charge states +2, +3, and +4 were written when charge state could not be auto determined. CID spectra from the same precursor were added and averaged to yield one spectrum if they fell within a mass window of 1.0 amu and a time window of 10 measurement cycles (maximum of 2.5 min). Spectra containing less than 10 peaks before treatment were discarded. Accepted CID spectra were processed as follows: peaks below 0.5% of the base peak were removed; remaining peaks were not smoothed but were centroided at 50% height with a merge distance of 2.0 amu. Collections of spectra were written as flat text files in Mascot Generic Format (.mgf).

Database Search. The UNIPROT (SWISSPROT release 46.0 +TrEMBL release 28.0, both of October 11th, 2004) protein

Table 1. Proteins Identified from Gel-based Separation of RelA-TA Affinity Experiment Using Nuclear Extracts from Three Cell Types (see Figure 1)[a]

band	acc. no.[b]	MW (kDa)	name	THP1 score[c]	JURKAT score[c]	HeLa score[c]	description	new?[d]	comment	gel comparison
1	no id									
2	*P78527*	*473*	*DNA-PKcs*	*175*	*137*	*1126*	*DNA-dependent protein kinase catalytic subunit*	*Y*	*PI3 protein kinase superfamily. DNA damage response*	*less in jurkat lane*
3+4	Q92793	268	CBP	137	675	245	CREB-binding protein	N	Histone acetyl transferase Transcription coactivator	ubiquitous
	Q09472	265	p300	229	594	212	E1A-associated protein p300	N	Histone acetyl transferase. Transcription coactivator	ubiquitous
	O75691	*318*	*DRIM*		*747*		*DRIM protein*	*Y*	*"Down regulated in metastasis" unknown function*	*only in jurkat lane*
5	*Q92616*	*293*	*hGCN1L1*			*403*	*KIAA0219 protein Human GCN1 homolog*	*Y*	*HEAT repeats*	*only in HeLa lane*
6	no id									
7	Q09472	265	p300			111	E1A-associated protein p300	N	degradation product of p300	more in HeLa
8	*Q86VI3*	*185*	*IQGAP3*	*69*		*67*[e]	*Ras GTPase-activating-like protein IQGAP3*	*Y*	*unknown function, contains GAP and IQ domain*	*in THP1, maybe HeLa, not Jurkat*
	P46940	*189*	*IQGAP1*			*67*[e]	*Ras GTPase-activating-like protein IQGAP1*	*Y*	*unknown function, contains GAP and IQ domain*	*only in HeLa*
9a	*Q9P0V5*	*148*	*MYBBP1A*	*187*	*773*	*351*	*MYB-binding protein 1A*	*Y*	*Binds and regulates transcription factors*	*more in jurkat lane*
9b	P53621	139	COPA	305		736	Coatomer alpha subunit	?	Probably nonspecific contaminant	ubiquitous

[a] See Table 3 in Supporting Information for raw data. [b] All database accession number are according to UNIPROT (SWISSPROT+TrEMBL). [c] MASCOT protein score. [d] Novel interacting proteins are italicized and indicated "Y" in the New? column (known interactors are indicated "N"). [e] Only peptides in common to the sequences of IQGAP1, IQGAP3 were matched, therefore it is impossible to discriminate between the two proteins.

sequence databases was used for all searches with the MASCOT 2.0 software (www.matrixscience.com[30]). Searching was limited to the *H. sapiens* subset, which contained 57 308 sequence entries. MASCOT search parameters were as follows: trypsin cleavage specificity with maximum one missed cleavage; carbamidomethyl cysteine as fixed modification, methionine single oxidation as variable modification, monoisotopic masses only were used, with peptide Mass Tolerance of ±0.8 Da and fragment mass tolerance of ±0.66 Da. The instrument type considered was ESI−QUAD-TOF. The standard (non "MUDPIT") MASCOT scoring scheme was used throughout the study. MASCOT was set up to only report peptide matches with a score above 14.

The value of 14 was chosen after manual evaluation of more than a thousand MS/MS spectra acquired under very similar conditions (same instrument, database, species, data handling) in the context of various research projects. We found this value to be a reasonable compromise between stringency and sensitivity of identification. Values below 10 for the threshold resulted in large numbers of false positive identifications. With the parameters used, the threshold for statistical significance ($p < 0.05$) corresponded to a total (protein) MASCOT score of 33.

Validation of Identifications. All protein identifications with a total MASCOT score below 80 were manually validated. Validation included examination of the peptide rms mass error (<120 ppm) and of individual peptide matches. Peptide matches with score between 14 and 40 were manually verified and validated only if at least an ion series of 4 consecutive y ions were matched, in addition to ions belonging to other series. Generally, only proteins matched by at least two peptides were accepted. Exceptions, i.e., single-peptide matches are labeled in Table 2 and are listed in the Supporting Information (Tables 3 and 4).

GST Pull Down with in Vitro Transcribed-Translated Mediator Subunits. DNA plasmids containing cDNA of the Mediator subunits were obtained from Image Consortium (cdk8, Med17/crsp6, Med7/crsp9, Med15/arc105) or kindly provided by Len Freedman and Christophe Rachez (Med24/drip100, Med14/drip150, Med1/drip205/trap220, Med23/drip130). Plasmids are based on pcDNA3.1 (Invitrogen) for all subunits except Med24 which is in pBluescriptII-sk+ (Stratagene). Mediator subunits were tagged with myc (cdk8, Med17/crsp6, Med7/crsp9, Med15/arc104, Med23/drip130, Med14/drip150) or flag (Med24/drip100) or untagged (Med1/trap220). ^{35}S-methionine labeled proteins were synthesized by use of TNT Quick coupled Transcription/Translation Systems kit following the manufactures protocol (Promega). Immobilized GST-RelA-TA or GST control were incubated with ^{35}S-methionine labeled Mediator subunits for 2 h at 4 °C with gentle rolling in buffer 20 mM HEPES-KOH pH 7.9, 10 mM $MgCl_2$, 0.1% NP40, 136 mM NaCl, 1.25% glycerol, 1 mM DTT, 0.2 mM PMSF, 1 μg/mL pepstatin, bestatin and leupeptin. Beads were then washed 3 times for 10 min with 0.8 mL buffer (20 mM HEPES-KOH pH 7.9, 10 mM $MgCl_2$, 0.1% NP40, 120 mM NaCl, 1 mM DTT, 0.2 mM PMSF, 1 μg/mL pepstatin, bestatin and leupeptin) and

Table 2. Proteins Identified by a Shotgun Approach in a RelA-TA Affinity Experiment using Nuclear Extracts from Three Cell Types[a]

acc. no	MW (KDa)	protein	THP1 score[b]	Jurkat score[b]	HeLa score[b]	function	new?[c]
P78527	473	DNA-PKcs	95	49	955	PI3K superfamily involved in DNA damage response	Y
Q9UIG2	313	Q9UIG2			66	Bromodomain PHD finger transcription factor	Y
Q92616	295	KIAA0219			914	hGCN1L1, hypothetical protein with HEAT repeats	Y
Q92793	268	CBP	236	261	587	histone acetyl transferase transcriptional coactivator	N
Q09472	266	p300	230	354	398	histone acetyl transferase transcriptional coactivator	N
Q8N3C0	252	HEL1	37			ASC-1 complex subunit p200	N
Q9UHV6	250	MED12	56			Mediator subunit transcriptional cofactor (trap230, drip/arc240)	N
P46940	189	IQGAP1			615	GTPase activating like protein IQGAP1	Y
Q86VI3	185	IQGAP3	301		44[d]	GTPase activating like protein IQGAP3	Y
Q15648	169	MED1	48[d]		48[d]	Mediator subunit transcriptional cofactor (trap220, drip/arc205)	N
O60244	162	MED14	68[d]			Mediator subunit transcription cofactor (trap170, drip/arc/crsp150)	N
Q9H0J2	158	MED23	95	44[d]		Mediator subunit transcription cofactor (trap150b, drip/arc/crsp130)	N
Q9P0V5	149	MYBBP1a	200	2124	726	Binds and regulates some transcription factors	Y
P53621[e]	*140*	*Coatomer alpha*	*773*	*349*	*260*	*ER-Golgi protein transport*	*na*[f]
Q9Y4C7	135	KIAA0690		67		HEAT repeats	Y
Q9UPN4	130	KIAA1118		49[d]		IQ domain	Y
Q6ZUM6	126	FLJ43536		46	44	hypothetical protein	Y
O75448	111	MED24	217	82	104	Mediator subunit transcriptional cofactor (trap/drip/arc/scrp100)	N
Q75T13	*106*	*PGAP1*		*33*[d]		*GPI inositol-deacylase PGAP1*	*na*[f]
P35606[e]	*103*	*Coatomer beta*	*847*	*422*	*515*	*ER-Golgi protein transport*	*na*[f]
Q6PJT2	94	MED16	88		46[d]	Mediator subunit transcriptional cofactor (trap95,drip92)	N
Q16891[e]	*84*	*Mitofilin*	*658*	*628*	*82*	*Mitochondrial inner membrane protein*	*na*[f]
Q6P143	78	MED25	48[d]			Mediator subunit transcriptional cofactor (arc92)	N
Q6IC31	75	MED15	41			Mediator subunit transcriptional cofactor (arc105)	N
P28288[e]	*75*	*ABCD3*	*226*	*653*		*Probable peroxisomal transporter*	*na*[f]
Q9NVC6	73	MED17	105	76		Mediator subunit transcriptional cofactor (trap80, drip/arc/crsp77)	N
Q6P1MO[e]	*72*	*Q6P1MO*		*61*		*Fatty acid transporter*	*na*[f]
Q9NVI7[e]	*66*	*FLJ10709*	*603*	*617*	*297*	*Hypothetical protein (ATAD3A protein)*	*na*[f]
Q96T67[e]	*65*	*TOB3*		*467*		*Unknown ATPase*	*na*[f]
Q9UNF1	65	MGD2			89	melanoma associated antigen D2	Y

[a] Keratins and well-known chaperones were removed from the list (see Supporting Information Table 4 for raw data). [b] MASCOT protein score. [c] Novel interacting proteins are indicated "Y" in the New? column (known interactors are indicated "N"). [d] identification based on one single peptide manually validated. [e] Entries in italic are likely non-specifically binding proteins (determined either by presence in the negative control or by an established nonnuclear intracellular localization). [f] na: not applicable (protein is either not specific or non nuclear).

proteins eluted from beads with SDS-laemmli buffer. Proteins were analyzed by 10% SDS-PAGE, gels were fixed for 30 min (50% methanol, 10% acetic acid), washed in water for 10 min 3 times, incubated for 30 min in Amplify (Amersham), dried and exposed to autoradiography film.

Results

Note: all database accession numbers are referred to Uniprot (AC field, www.expasy.org).

Different Pattern of RelA-TA Interacting Proteins is Seen from THP1, Jurkat and HeLa Nuclear Extracts. To identify new cell type specific cofactors of RelA, we tried to find cofactors from different human cell types that have the ability to interact with the transcription activation (TA) domain of RelA/p65. Since the TA domain of RelA is the domain responsible for transcription activation and binds to known co-activators of transcription, this domain was used as it is most likely to interact with other unknown transcription cofactors. We performed GST pull down experiments using GST-RelA-TA (amino acids 431−551). Immobilized GST-RelA-TA or an equal amount of GST control was incubated with up to 10 mg of nuclear extracts from 3 different immortalised cell types (epithelial − HeLa S3, T cells − Jurkat, and monocytes − THP1) responsive to NF-κB activating signals. Bound proteins were analyzed by 1D gradient SDS-PAGE, stained with SyproRuby and visualized with a Typhoon scanner under a blue light (488 nm) laser source (Figure 1). The advantages of visualizing proteins with this fluorescent dye is that it binds noncovalently to proteins (enabling efficient analysis by mass spectrometry) and binds quantitatively to proteins (allowing relative quantification of the proteins bound to GST-RelA-TA). Since the molecular weight region of the gel below 60 kDa is dominated by protein bands related to GST-RelA-TA, we choose to concentrate on the high molecular weight region. We therefore used a 3−8% gradient gel for protein separation. The pattern of RelA-TA binding proteins from the nuclear extracts of the three different cell types is similar with some differences, which could be due to the presence of cell type specific or enriched RelA-TA binding proteins (Figure 1 and Figure 2C). Results from two separate experiments were compared and the general pattern of the bands was similar (Figure 2A). However, there were some differences of the relative intensity of each band between experiment 1 and 2 (Figure 2B).

Identification of Novel RelA-TA Binding Proteins. Protein bands seen in the GST-RelA-TA bound lane and not in the GST control lane are labeled in Figure 1. These proteins are considered to be interaction partners of RelA-TA. Band 9 is a doublet, where the lower band (9b) appears to be present in the negative control while the higher band appears to be a specific RelA-TA interactor. We systematically analyzed all proteins in individual bands by in-gel tryptic digestion and liquid chromatography-tandem mass spectrometry (LC−MS/

MS). Results are summarized in Table 1, which indicates RelA-TA binding proteins with a brief description of their known or putative function. The new RelA-TA binding proteins found with this approach are DNA-PKcs, DRIM, KIAA0219 (hGCN1L1), IQGAP3, and MYBBP1a. In a separate affinity experiment carried out with nuclear extracts of HeLa cells in the absence of ethidium bromide (data not shown), we were able to identify three additional proteins, TRRAP (Q9Y631, score 308), the protein kinase ATR (Q13535, score 125), and a hypothetical protein containing a YEATs domain, a typical feature of proteins with transcription stimulating activity (KIAA1197, score 108).

To confirm the findings and attempt to find all possible potential interaction partners of RelA-TA, we further analyzed the samples with a shotgun approach. We separated the protein mixtures eluted from glutathione sepharose beads on a 7%, 1.7 cm minigel, cut each lane into 5 segments corresponding to varying molecular weight ranges from 60 to ~400 kDa and analyzed each segment containing a group of proteins by digestion and LC−MS/MS (Table 2). The resulting collections of CID spectra were pooled for each sample and used for a single database search, yielding a list of identified proteins per cell line. Table 2 presents a comparative view of this shotgun experiment. All but one (DRIM) of the proteins listed in Table 1 were also identified by this technique, thus confirming the results of the gel approach. Disregarding keratins and well-known chaperones, 23 additional proteins were found (compared with the band-by-band approach), among which 9 components of the Mediator complex and several hypothetical proteins. These results emphasize the higher performance of such a technique for the purpose of protein identification. Some of the ubiquitous proteins found were probably non-specific contaminants, such as mitofilin (a mitochondrial membrane protein) and Coatomer binding proteins A and B (COPA was seen in the GST negative controls in SDS-PAGE at 140 kDa). Several other identifications were based on relatively low scores and were carefully validated by manual inspection of MS/MS data.

Identification of Cell Type Enriched RelA-TA Binding Proteins. We identified proteins present in the GST-RelA-TA bound lane at a much higher level in certain cell types. These proteins are labeled with stars (Figure 1) and are considered to be cell type enriched interaction partners of RelA-TA. Due to comigration of several proteins in some bands and high background levels, it was difficult to obtain accurate protein quantification by densitometry (Figure 2C). Nevertheless, we found that MYBBP1a (band 9a), although found in all cell types, is a Jurkat enriched binding partner of RelA-TA, while hGCN1L1 (band 5) is a highly HeLa enriched binding partner of RelA-TA. IQGAP3 and/or IQGAP1 (band 8) were identified as RelA-TA binding partners from THP1 and HeLa but not Jurkat nuclear extract.

The shotgun identification round (results in Table 2) confirmed and complemented the gelband-based results. For low scoring proteins, it is impossible to draw any conclusion on the relative abundance of specific proteins binding to RelA-TA from the nuclear extracts of the three cell types examined, because of the limited reproducibility of the sampling by LC−MS/MS for low-intensity signals and one pass analysis.[31] It has been previously shown[31] that the sampling of complex mixture of peptides is not complete and that, depending on complexity, repeated analyses are needed to efficiently capture and identify most peptides in a mixture. On the other hand, Liu et al.[31] also showed that sampling levels (i.e., number of matched peptides) can be taken as a good measure of protein abundance over at least 2 orders of magnitude. For high scoring proteins the results correlated well with gel-based quantifications. KIAA0219/hGCN1L1 was found in this approach to bind to RelA-TA specifically from the nuclear extracts of HeLa cells. IQGAP1 and its close homologue IQGAP3 were not detected to bind to RelA-TA from the nuclear extracts of Jurkat cells but were found to bind from the nuclear extracts of HeLa and THP1 cells, respectively. MYBBP1a was found to be a RelA-TA binding protein from the nuclear extracts of all three cell types, however with a much higher score from Jurkat than HeLa or THP1 nuclear extracts, again supporting the data from the first gel-based approach. Although two members of the Mediator complex (Med12 (trap230, drip240) and Med14 (trap170, drip150)) were found exclusively in RelA-TA binding proteins from THP1 monocyte nuclear extract samples, they had a low score so we cannot exclude the possibility that they were present at low levels in the other samples. In a separate experiment with HeLa cells (not shown), we found band 5 to contain, in addition to hGCN1L1, the mediator subunit Med12 (trap230). This indicates that the weak band 5 in THP1 and Jurkat lanes could indeed correspond to Med12 (which was detected in THP1 by the shotgun approach) however in quantities too low for detection after in-gel digestion.

Surprisingly, DRIM, which according to the gel experiment is a Jurkat specific RelA-TA interacting protein, was not identified in this analysis. It is most likely that DRIM was present in the shotgun approach, but was undetected. This could be due to the mini-gel system (used for the shotgun approach) not being ideal for the detection of very large proteins (>300 kDa). Consistent with this, in two (Jurkat, THP1) of the three samples the scores for the very large protein DNA-PKcs were lower in the shotgun approach than what was usually seen for the same protein in the band-by-band approach using a gradient gel. This could be due to the short migration time of the minigel, possibly leading to a proportion of some high MW proteins not entering into the gel. Alternatively, a difference in the efficiency of the digestion procedure between the 2 gel types could explain a lower score for the very high molecular weight protein fractions in the shotgun approach. However, since DRIM has been identified twice in previous gel-based experiments, its presence seems to be reproducible.

MED14 (drip150/trap170), MED17 (drip77/trap80), and Med24 (drip100/trap100) Specifically Interact Weakly with RelA-TA. It has been shown before that RelA-TA interacts with the Mediator complex,[32] and this is confirmed by our results above (Table 2). We found 9 Mediator subunits binding to RelA-TA, however all peptides were found with low scores. We tried to confirm the interaction by analyzing which of the Mediator subunit(s) could interact with RelA-TA specifically. We transcribed and translated in vitro in the presence of ^{35}S methionine all available Mediator subunits (8 in total including 6/9 subunits found here) and incubated each separately with GST-RelA-TA or GST control. We found a very weak interaction with Med14 (drip150/trap170), MED17 (drip77/trap80) and Med24 (drip100/trap100) (Figure 3).

Discussion

Each cell type is responsive to a specific pattern of NF-κB activating stimuli to give a cell type specific pattern of NF-κB dependent gene expression. This specificity can occur through several mechanisms as different cell types could have specific

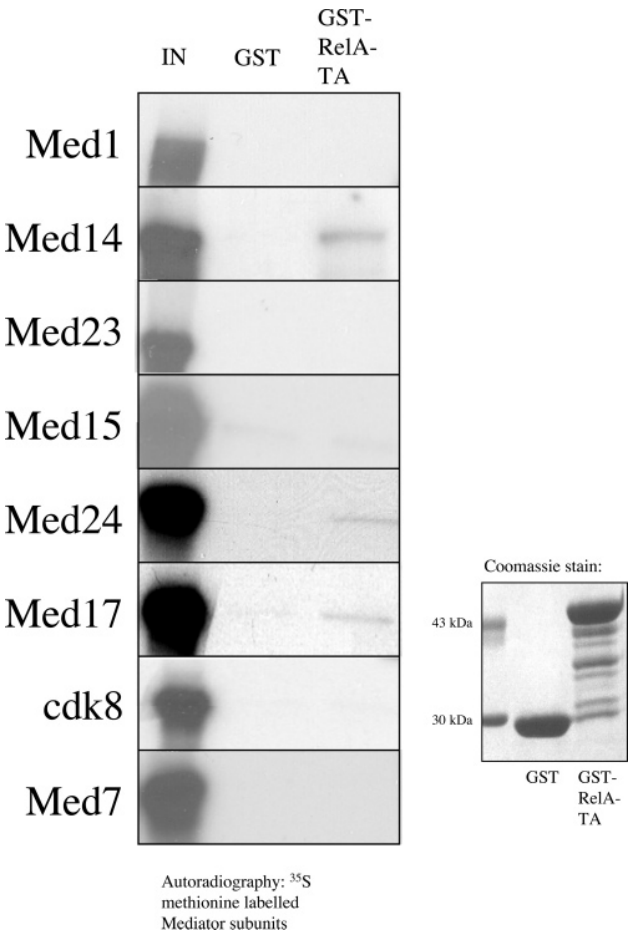

Figure 3. Mediator subunits (Med14, Med17, Med24) interact weakly with RelA-TA. ^{35}S methionine labeled Mediator subunits (cdk8, Med17/crsp6, Med7/crsp9, Med15/arc105, Med24/drip100, Med14/drip150, Med1/drip205, Med23/drip130) were synthesized from rabbit reticular lysate using TNT Quick (Promega). Mediator subunits were incubated with immobilized GST-RelA-TA or GST control, bound proteins were washed then analyzed by SDS-PAGE and autoradiography. Inputs are 4% of total protein used in each pull down. GST and GST-RelA-TA inputs are shown (below, right).

(a) surface receptors and signaling pathways (b) expression levels and post-translational modification patterns of each NF-κB dimer (e.g., RelA/p50, RelB/p52, c-rel/c-rel etc.) that have different promoter/enhancer binding preferences (c) modification patterns within the chromatin resulting in different regions of the genome being accessible for transcriptional control, and (d) active cofactors that are able to bind to NF-κB to regulate subsets of genes. This could be due to differences in the expression levels or activity of cofactors in the different cell types or differences in the NF-κB binding potential of a cofactor from different cell types. To investigate this, we identified and compared the pattern of proteins that could bind to the GST-RelA-transcription activation domain (TA) from the nuclear extracts of 3 different immortalised cell types (epithelial-HeLaS3, T cell- Jurkat, and monocyte-THP1). It was considered useful to use high amounts of the TA domain as no known RelA-TA interacting transcriptional cofactors were found in an extensive study that identified RelA interacting proteins by co-immunoprecipitation of full length TAP tagged RelA from 293 HEK cells.[33] Nuclear extracts were used as it is thought that most cofactors of transcription are found predominantly in the nucleus. Interacting proteins were identified by electrospray mass spectrometry.

New RelA-TA Interacting Proteins. Many proteins were found in this study that have previously been reported to interact with RelA-TA. These are p300/CBP[7] and subunits of the Mediator complex.[32] Also found here were many new interacting proteins of RelA-TA, which are described in detail below. These proteins were not found in the co-immunoprecipitation study mentioned above (although a member of the IQGAP family was found to bind to RelA), where mainly cytoplasmic molecular partners were found. The RelA-TA that we use in these experiments was expressed and purified from bacteria and therefore does not contain the same pattern of post-translational modifications that a cellular transcriptionally active RelA-TA is expected to contain. Therefore, other RelA cofactors that interact with RelA-TA in a way dependent on post-translational modifications may not have been detected in this study. It is also important to consider that the approach used here cannot distinguish between proteins that directly interact with RelA-TA and those that are pulled down in a complex with a RelA-TA binding partner (second-order interactions).

1. DNA-PKcs, ATR, and TRRAP. DNA-PKcs (DNA-dependent protein kinase catalytic subunit), ATR (ataxia telangiectasia mutated and Rad3 -related protein) and TRRAP (transformation-transactivation domain-associated protein) are proteins of the phosphatidylinositol kinase (PI3K) superfamily, which contain a PI-3 like kinase domain. DNA-PKcs and ATR, together with another member of this family, ATM (ataxia telangiectasia mutated), are serine/threonine kinases recognizing the consensus motif Ser/Thr-Gln-Glu.[34] They are activated in the nucleus early during cellular genotoxic stress, and phosphorylate many proteins involved in cell cycle checkpoints, DNA repair and apoptosis. One of their targets is the transcription factor p53, which is phosphorylated and activated by ATM, and possibly also by ATR and DNA-PKcs in response to genotoxic stress such as gamma irradiation, UV, heat shock, and nitric oxide.[35−38] NF-κB may be involved in the response to these signals as NF-κB binding sites have been found in genes regulated by many of these genotoxic signals. DNA-PKcs and ATM have been shown to regulate NF-κB dependent transcription by the direct or indirect phosphorylation of IκBα in response to DNA damage which resulted in IκBα degradation and activation of NF-κB DNA binding.[39,40] Here, we show that the RelA-TA can bind to the kinases DNA-PKcs and ATR. This indicates that the regulation of NF-κB dependent transcription by genotoxic stress could happen directly through the RelA subunit as well as via phosphorylation of IκBα. DNA-PKcs and ATR may also have general roles in transcription. Cell extracts lacking DNA-PKcs were deficient in multiple rounds of transcription as assayed by in vitro transcription on a supercoiled DNA template.[41] ATR is part of the NRD (nucleosome remodeling and deacetylating) complex which contains HDAC2.[42]

TRRAP was found to be a member of four different histone acetyltransferase complexes, STAGA (contains GCN5-L),[43] PCAF (contains P/CAF),[44] TFTC (contains GCN5-L),[45,46] and NuA4 (contains tip60).[47] This indicates that TRRAP, which has no known kinase activity, has a role in DNA metabolism processes that require remodeling of chromatin. A microarray gene expression comparison between TRRAP conditional knockout mouse embryonic fibroblast and TRRAP containing cells indicated that TRRAP is responsible for the regulation (usually

activation) of specific sets of genes.[48] TRRAP has been shown to bind to the transactivation domains of c-myc and E2F and the TRRAP/c-myc interaction is necessary for transcription of a subset of proliferation genes such as cyclinD2 and TERT, but not for other basally expressed genes.[49] Other transcription factors such as p53 and the oestrogen receptor require HAT complexes containing TRRAP to transcribe specific genes.[50,51] Since RelA is known to be acetylated by P/CAF,[52] and can interact with TRRAP (shown here) it may be that recruitment of P/CAF to RelA could be via the TRRAP component of the PCAF complex.

2. MYBBP1a. MYBBP1a (p160) and a related protein (p67) were first identified by their ability to bind the leucine zipper region within the negative regulating domain of the c-Myb proto-oncogenic transcription factor.[53] p67 was found to be an amino terminal fragment of MYBBP1a formed by proteolytic cleavage only in some cell types and although both p67 and MYBBP1a could bind c-myb, only full length MYBBP1a could bind the Jun transcription factor.[54] Here we find that MYBBP1a can also bind to the RelA-TA but since we were focusing on RelA-TA binding proteins that were above 100 kDa we did not test whether p67 could also bind. Expression of a short version of MYBBP1a (approximating p67) could repress c-myb dependent transcription in a reporter assay, whereas MYBBP1a had little affect.[54] Interestingly, MYBBP1a was found to be predominantly nucleolar.[54] It can also interact with the acidic activation domain of AhR transcription factor and activates transcription of a AhR responsive reporter gene.[55] MYBBP1a was found to interact with the negative regulatory domain of PGC-1α co-activator of transcription, and inhibit its activity.[56] The mechanism of transcriptional regulation is not known. The interaction between RelA-TA and MYBBP1a indicates the possibility that MYBBP1a might affect NF-κB dependent gene expression.

3. IQGAP1 and 3. IQGAP1 protein is a GTPase activating protein (GAP) with IQ (calcium independent calmodulin binding) domains. It is thought to function in the cytoplasm or plasma membrane, although a nuclear localization has not been disproved. IQGAP1 is involved in the negative regulation of cell−cell adhesion through Rac1 and Cdc42 GTPases.[57] It is up regulated in some cancer cell lines and promotes cellular invasion.[58] We identified IQGAP1 as a possible interacting partner of RelA-TA, although since it is thought that the proteins localize to different cellular compartments, this interaction seems unlikely to occur in vivo. However, it has also been found that IQGAP1 can activate transcription by the plasma membrane/cytoplasm/nuclear shuttling transcription factor beta-catenin,[59] suggesting that interaction with other transcription factors could be possible. IQGAP3 is 58% identical (75% similarity) to IQGAP1 and up regulated in gastric cancers. Nothing is known about its cellular localization or its function. Previously, IQGAP2 was found to bind to full length RelA in a large study of the NF-κB pathway, indicating that members of the IQGAP family could be NF-κB interactors.[33]

4. DRIM. DRIM (down regulated in metastasis) was found by comparison of nonmetastatic breast cells with metastatic cells.[60] The function or subcellular localization of DRIM is not known, although it contains HEAT repeats that occur in many intracellular transport proteins. Interestingly, ATR and KIAA0219 (hGCN1L1) which we also found to interact with RelA-TA, contain HEAT repeats.

5. KIAA0219 − Human GCN1L1. KIAA0219 − Human GCN1L1 (GCN1 general control of amino acid synthesis 1-like 1) is a hypothetical protein.[61] It contains HEAT repeats and 3 ARM (Armadillo/beta-catenin-like) domains. By sequence alignment, this protein is believed to be the human counterpart of the yeast GCN1 translational regulator. However, yeast GCN1 contains 30 HEAT repeats, while the human KIAA0219 only 3. No other information on the localization or function of this protein is available.

Cell Type Enriched RelA-TA Interacting Proteins. In this study, we compare RelA-TA binding proteins from 3 different cell types (Figure 1, Tables 1 and 2). Since all cell lines used were transformed cell lines, we cannot distinguish between RelA-TA binding proteins enriched due to the cell type or due to the different transformation process that have occurred within each cell type. However, we consider both groups of proteins interesting for future studies. It is possible that the proteins enriched in the various cell types, listed below, are involved in the differential transcription regulation by RelA/p65 seen in these different cell types. The differences in the recovery of these proteins bound to RelA-TA may be due to differences in overall expression levels or due to specific targeting to RelA-TA in the different cell types. To distinguish these possibilities, it would be necessary to quantify the expression levels of these proteins in the different cell types, however this could not be determined due to the absence of commercially available antibodies. Measurement of mRNA levels could give an indication of the relative expression levels in the three cell types studied.

1. IQGAP1/3. The combined evidence from both the band-by-band and the shotgun approach indicate that IQGAP3 is uniquely recovered in THP-1 samples, while IQGAP1 is predominant in HeLa. None of these proteins was detected in samples from Jurkat cells. Nothing is known about the function, expression pattern, or cellular localisation of IQGAP3.

2. MYBBP1a. More MYBBP1a was found to interact with RelA-TA from Jurkat nuclear extract than from HeLa or THP1 nuclear extract. MYBBP1a transcripts were expressed in all mice tissues and human cell lines tested, although the abundance of the transcript varied.[54,62] This could indicate that MYBBP1a is a Jurkat enriched interactor of RelA-TA. Our future work will be the investigation of MYBBP1a as cell type specific RelA cofactor.

3. KIAA0219 (hGCN1L1). We found more of the hypothetical protein KIAA0219 bound to RelA-TA from HeLa nuclear extract than from Jurkat or THP1 extract. We therefore provisionally name this protein RELAH (standing for Rel A binding protein from HeLa).

Confirmation of Mediator Interaction with RelA-TA. In the shotgun approach (Table 2) we find totally 9 subunits of the Mediator that bind to RelA-TA. Only high molecular weight proteins (>60 kDa) were analyzed so we have no information for the lower molecular weight Mediator proteins. These data agree with the previous report of Mediator interacting with RelA-TA.[32] The Mediator complex is composed of 4 domains (head, middle, tail, and cdk8 domain) and is thought to regulate transcription (positively and negatively) through interaction of polII and DNA binding transcription factors (reviewed in ref 63). We found subunits from all domains of the Mediator complex, increasing the likelihood that the whole Mediator complex binds to RelA-TA in the conditions tested. Analysis of the interaction of individual in vitro translated Mediator subunits with RelA-TA indicates Med14 (trap170/drip150), Med17 (trap80/drip77), and Med24 (trap100/drip100) have the ability to interact weakly with RelA-TA. We tested the 8 Mediator subunits available (cdk8, Med17/crsp6, Med7/crsp9,

Med15/arc105, Med24/drip100, Med14/drip150, Med1/drip205, Med23/drip130) leaving the possibility remaining that other subunits also interact with the RelA-TA. The weakness of the interactions observed could be due to the need for other domains of RelA or for several Mediator subunits to be present in order to form a high-affinity binding complex.

Methodology, Performance and Confidence of the Identification Results. Despite the use of a relatively large amount of bait protein and cell extract, the quantity of proteins purified for analysis was medium to very low. This could be due to the fact that transcription activators and cofactors are often expressed at low levels in most cells and our washes were very stringent. The gel separation analysis offers the possibility to compare band patterns among samples from different cell lines as well as relative to a negative control, thus verifying the specificity of the bands analyzed. However, this approach is not comprehensive, as bands close to or just below the detection sensitivity are neglected. In addition, we found in-gel digestion with acrylamide bands below 6% percentage to yield poorly reproducible results, thus making comparisons of protein identifications among bands difficult. The shotgun approach we have adopted subsequently is faster and results in a more complete mapping of the global composition of the samples. We also found this method to be more sensitive since the limited migration increases the protein concentration in the gel resulting in better digestion yields. Ideally, shotgun experiments should be performed without gel separation (in liquid phase), to ensure reproducibility of the digestion conditions and comparability between experiments. However, in our situation the partial separation offered by the gel step allowed the elimination of a high level of background due to the GST-RelA-TA fusion protein (approximately 10 μg/sample). In addition the SDS extraction is very efficient to detach all proteins recovered in the affinity step. However, no information is obtained about the relative quantities of proteins in different samples for low-scoring identifications, especially since the shotgun experiment was performed only once due to the limited amount of sample available. To improve the shotgun technique, a strategy allowing accurate quantification of proteins present in low amounts should be developed and used, such as the multiplexed labeling proposed recently.[64] However, this was not available during this study.

Contaminant and Non-physiological Interacting Proteins. Several proteins were detected in the shotgun experiment, for which we have no information on possible specificity of their interaction with RelA-TA. Some proteins with a well-characterized cytoplasmic or nonnuclear location (e.g., mitochondrial) can be ruled out as in-vivo binding partners of RelA. These include the coatomer binding proteins and mitochondrial or peroxisomal proteins (Q16891, P28288, and Q9NVI7). Well-known heat shock proteins, chaperones, and keratins were found and are not listed in Table 2 since they are ubiquitous in pull-down experiments (see Supporting Information, Table 4). For other poorly characterized molecules such as the melanoma-associated antigen Q9UNF1 found in HeLa cells, precise quantitative data relative to its presence in the negative control are necessary to discern about its specificity of interaction with the bait protein (RelA-TA). The possibility that DNA-PKcs, ATR, and TRRAP were contaminating proteins in the pull down experiments cannot be disregarded. Since DNA-PKcs and ATR can bind directly to DNA, if small amounts of DNA were present in the nuclear extract and other RelA-TA interacting proteins could bind to the DNA, this could lead to contamination of the RelA-TA bound sample with DNA binding proteins. However, this is unlikely as washes were extensive and stringent and no other general DNA binding proteins were found. Also, ethidium bromide (preventing most DNA-protein interactions) was used in most pull downs including those where DNA-PKcs was detected.

Conclusions

The technique used here was successful in identifying new, and also cell type enriched RelA-TA interacting proteins. Further investigations of the cellular localization pattern, cell type expression pattern, and effect on RelA dependent transcription of these proteins are necessary to establish their role as cofactors of RelA-dependent transcription.

Abbreviations: ATR, Ataxia telangiectasia mutated and rad3 related protein; CBP, Creb binding protein; CID, Collision induced dissociation; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; HATs, Histone acetyl transferases; LC−MS/MS, Liquid chromatography-tandem mass spectrometry; MYBBP1a, Myb binding protein 1a; NF-κB, Nuclear factor kappa B; TA, Transcription activation domain; TRRAP, transformation−transactivation domain-associated protein.

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Supporting Information Available: Single-peptide matches and raw data, containing well-known heat shock proteins, chaperones, and keratins, are provided in Supporting Information Tables 3 and 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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MYBBP1a is a novel repressor of NF-κB

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Short Title: MYBBP1a is a novel repressor of NF-κB

Synopsis

NF-κB is an inducible transcription factor activated in many different cell types by inflammatory and stress signals. The transcription of a wide variety of NF-κB genes is regulated by the coordinated action of transcription co-activator and co-repressors. Previously we identified Myb binding protein 1a (MYBBP1a) as an interaction partner of the transcription activation domain of RelA/p65. MYBBP1a has been shown by others to regulate various transcription factors, through largely unknown mechanisms. Here we present evidence that MYBBP1a is a novel co-repressor of NF-κB. Expression of MYBBP1a in cells repressed NF-κB dependent reporter expression. *In vitro*, MYBBP1a inhibited transcription from chromatinised templates at a step before pre-initiation complex formation. MYBBP1a was found to compete with the histone acetyl transferase co-activator, p300, for interaction with the transcription activation domain of RelA/p65. Expression levels of MYBBP1a are dependent on the cell type, and are particularly high in Jurkat T cells. These results indicate that MYBBP1a is a novel NF-κB co-repressor of transcription that competes with p300 and may function to regulate cell type specific genes.

Keywords

NF-kappa B, RelA, p65, transcription, co-repressor, MYBBP1a

Abbreviations

HDAC, histone de-acetylase; IκB, inhibitor of NF-κB; LTR, long terminal repeat; MN, micrococcal nuclease; MYBBP1a, Myb binding protein 1a; PMA, phorbal 12-myristate 13-acetate TAD, transcription activation domain.

Introduction

RelA (also called p65) is a member of the NF-κB family of inducible transcription factors (reviewed in [1]). Other members of this family are c-Rel, RelB, p105/p50 and p100/p52. NF-κB regulates the expression of a wide variety of human genes including cell cycle regulators, apoptosis factors, proliferation inducers and immuno-regulatory proteins (reviewed in [2]). There are also NF-κB response elements in the regulatory regions of viruses, such as in the Long Terminal Repeat (LTR) of HIV-1 [3]. NF-κB activity is aberrantly regulated in many diseases, including cancer (reviewed in [4] and [5]). For these reasons many aspects of the regulation of transcription activation by NF-κB are well studied.

RelA/p65 can be regulated in both positive and negative ways. The "inhibitor of NF-κB" (IκB) family of proteins are major inhibitors of NF-κB transactivation of target genes. In most cells, the majority of NF-κB is found in the cytoplasm in a complex with IκB. Once the appropriate cell is stimulated by a specific signal, phosphorylation and activation of the IκB kinase complex is triggered, leading to IκB phosphorylation, ubiquitinylation, and finally degradation. This results in the accumulation of RelA/p65 in the nucleus (for recent reviews see [6] and [7]). RelA/p65 can also be repressed in the nucleus, by association with IκB proteins [8-10] or transcriptional co-repressor histone deacetylase complexes [11-13].

RelA/p65 contains a C-terminal acidic transcription activation domain (TAD) consisting of TA1 and TA2 sub-domains [14]. The TAD contains conserved amino acid motif repeats and has been described as a random coil by NMR studies [15]. However, TA1 peptides in a hydrophobic solution were measured by circular dichroism to have alpha helical properties [15], indicating an induced structure may be formed when the TAD is part of a higher order complex. The TAD of RelA/p65 can be modelled as an alpha helix, and this model has features highly conserved between species. Five positions in the modelled helix consist of hydrophobic amino acids and these are interspersed mainly with acidic residues [16]. On the opposite side of the modelled helix is a region of conserved serines. At least three of these serines (S529, S536 and S468) are inducibly phosphorylated, forming negatively charged patches on this surface, and leading to enhancement of the transcription potential of RelA/p65 ([17, 18] and reviewed in [19]). Many groups have identified different proteins that bind to the TAD of RelA/p65 and may form a variety of higher order

protein complexes with different influences on the transcriptional potential of RelA/p65 [20-26].

Previously we identified MYBBBP1a as an interacting partner of the TAD of RelA/p65 [20]. Higher levels of MYBBP1a were found to bind to the TAD from Jurkat (T cell) nuclear extracts than from THP1 (monocyte) or HeLa (epithelial) nuclear extracts. MYBBP1a was first identified as a protein that could interact with the negative regulatory region of the transcription factor Myb [27]. It is a 150 kDa protein encoded by a single 4.5 kbp mRNA expressed to varying levels in all cell lines and mouse tissue tested. In some cell types an N terminal fragment of MYBBP1a (p67) was also found [27]. MYBBP1a was shown to be present mainly in the nucleolus in NIH 3T3 fibroblasts and 293T cells [27, 28]. Its role in the nucleolus is unknown but is consistent with the nucleolar localisation of a *S. pombe* homologue called POL5 [29]. Over a region of 880 amino acids, MYBBP1a and POL5 are 21% identical, and 41% similar. MYBBP1a and POL5 share an acidic domain of unknown function and LXXLL motifs that often mediate interactions with nuclear hormone receptor transcription factors (reviewed in [30]). However, MYBBP1a does not contain any polymerase domains that were found to be active in POL5. Unlike other DNA polymerases, POL5 has an important role in rRNA transcription [29]. This indicates MYBBP1a might also have a role in rRNA transcription by pol I in the nucleolus, but this has not yet been investigated. However, there is some evidence that MYBBP1a has a role in pol II dependent transcription regulation. It has been shown to bind to the transcription factors Myb [27], c-Jun [27], AhR [31] and PGC-1α [32], although the influence of MYBBP1a on transcription was variable.

Here we investigate the role of MYBBP1a on NF-κB dependent transcription. MYBBP1a was found to interact with the transcriptional activation domain (TAD) of RelA/p65, and this interaction is likely to be direct. In transient transfection reporter assays MYBBP1a repressed transcription from the NF-κB responsive promoter HIV1-LTR. We found that MYBBP1a is present in Jurkat (T) cells at higher levels than in THP1 (monocyte) or HeLa (epithelial) cells. Consistent with previous reports, we found myc-tagged MYBBP1a in the nucleolus of Jurkat (T) cells. MYBBP1a competed with the co-activator p300 for interaction with RelA/p65-TAD. *In vitro* transcription experiments with "chromatinised templates" as the substrate for transcription showed that MYBBP1a could inhibit transcription activated by

RelA/p65 and p300. MYBBP1a was able to inhibit transcription at a stage after RelA/p65 binding to DNA and before pre-initiation complex formation. These results indicate that MYBBP1a might be enriched in T cells and is a co-repressor of NF-κB dependent transcription, by competing with the co-activator p300.

Experimental

Plasmid DNA

The NF-κB dependent luciferase reporter constructs pGL2-HIV1-LTR-WT and pGL2-HIV1-LTR-ΔκB(MUT) are described in [33]. The p53 dependent reporter constructs BS2-WT and BS2-MUT were described in [34]. Expression plasmid for p53 and p65 were based on pcDNA3 and were described in [35]. Expression plasmid for MYBBP1a (pcDNA3kmMYBBP1a) was constructed by PCR amplification of MYBBP1a from the IMAGE clone 5495483. The forward primer 5' TAT AAG CGG CCG CGA TGG AGA GCC GGG ATC CC 3' and reverse primer 5' TAT AAT CTA GAC TAT CAG GGC TTC CCT GCC TTC C 3' allowed Not1/Xba1 digestion and insertion into a pcDNA3 based vector with a myc-tag in frame. The resulting construct was sequenced and the NT amino acid sequence consisting of the first methionine, the myc tag polylinker and the first methionine of MYBBP1a is MEQKLISEEDLKLIDGSRAAAM. Expression in 293T was tested after transient transfection of the construct.

Antibodies

Anti-p65 C20, anti-PCNA PC10 and anti-p300 C20 were purchased from Santa Cruz, anti-his (tetra-his mouse monoclonal IgG1 antibody) from Qiagen, anti-PARP from Cell Signalling, anti-tubulin from Sigma, and anti-c-myc antibody from Roche. Whole serum for anti-MYBBP1a was a kind gift from Rebecca Keough (School of Molecular and Biomedical Science, University of Adelaide).

Transient transfection reporter assays

Jurkat cells (EAECC) were maintained in RPMI 1640 medium + L-glutamine (Gibco), 10 % fetal calf serum, penicillin and streptomycin. 5-10 million cells were used per sample, and samples were always in duplicate. Transfection was performed according to the standard DEAE-dextran. DNA was purified with the Quiagen ENDO free maxi prep KIT. 2 μg of reporter plasmid DNA was used, with the indicated amounts of test plasmid DNA. Control plasmids (empty vectors) were used in the appropriate amounts to keep the number of each promoter constant in each sample, to avoid promoter squelching. Stimulation with 17.5 nM PMA (phorbal 12-myristate 13-acetate purchased from Alexis Biochemicals) was for 4-5 hours. After transfection the

cells were left 24-36 hours, before being harvested, washed with PBS and lysed in Reporter lysis buffer (Promega). Luciferase levels were measured using Luminol substrate (Promega) and the Lumat LB 9507 luminometer. The luciferase levels were normalised against the protein concentration of the extracts, and the mean was taken of the duplicate samples. The experiments were repeated three times, and the mean of the three experiments was taken, and the standard deviation calculated.

GST pull down experiments

GST-pull downs were performed under standard conditions. Bacterial extracts (GST or GST-RelA/p65-TAD) or Sf21 extracts (GST-RelA/p65) expressing the GST fusion protein of interest were incubated with rolling 1 h, 4°C, with Glutathione Sepharose beads (Amersham) in GST binding buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 10 % glycerol, 0.1% NP40, 1.5 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, PLB). Immobilised GST proteins were washed with GST wash buffer 3 times (20 mM HEPES pH7.5, 380 mM NaCl, 10 % glycerol, 1.5 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, PLB) and then 1 time with MYBBP1a binding buffer (20 mM HEPES pH 7.5, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, PLB, 50/80/120 mM NaCl as indicated in the figures. Proteins (his-MYBBP1a or his-p300 or BSA) were added to the binding reaction of total volume 500 µl and incubated with rolling 2 hr 4°C. In figure 1D and E this brought the salt conditions to 97/135 mM NaCl. Immobilised proteins were washed in MYBBP1a binding buffer 3 times, and denatured with 10X SDS loading buffer. Proteins were analysed by SDS-PAGE and either coomassie staining or western blotting. For the reactions with micrococcal nuclease digestions the basic prototol from Nguyen 2006 was followed [36]. All proteins were MN treated (GST proteins when immobilised) with 12U/100 µl reaction MN (MBI) in MYBBP1a binding buffer supplemented with 5 mM $CaCl_2$ at 30°C 10 min.

Nuclear Extract Preparation

Following the modified Dignam method [37] described in full previously [20]. Cells were stimulated with 175 nM PMA (Alexis Biochemicals) for 1 hr.

Immunoflourescence

Jurkat cells were transfected with the same number of molecules of MYBBP1a expression plasmid or empty vector control corresponding to 5 μg pcDNA3kmMYBBP1a or 2.87 μg pcDNA3 using the DEAE-dextran method. After 40 hr, cells were treated for a total of 1 hr at 37 °C with 17.5 nM PMA or 175 nM PMA (Alexis Biochemicals) or 30 ng/ml TNF-α (Sigma). Cells were attached to poly-L-lysine (Sigma) coated chamber slides (LabTech) at 37 °C for the last 30 min of stimulation. Cells were fixed with 4% formaldehyde in PBS 20 min, room temperature, rocking gently. Fixed cells were washed 2 times, 5 min with PBS, permeabilised with 0.2 % triton X 100 in PBS, 10 min and washed again. Slides were blocked in 2% BSA in PBS 1 h, washed, incubated with anti-myc antibody (Roche), washed, incubated with anti-mouse-FITC antibody (Jackson Immunology) and washed. Cells were stained with DAPI, washed, and mounted with Vector Shield (Vector Laboratories).

Expression and Purification of his-MYBBP1a, his-p65 and his-p300

His-MYBBP1a was expressed in insect Sf21 cells using the BacPAK Baculovirus Expression System (Clontech). MYBBP1a was PCR amplified from the IMAGE clone 5495483 using a forward primer containing a 6X his tag and XhoI restriction site 5' AAT ACT CGA GAC CAT GCA CCA CCA TCA CCA CCA TGG TAT GGA GAG CCG GGA TCC C 3'. The reverse primer contained 2 stops and a NotI restriction site 5' TAA TAG CGG CCG CCT ATC AGG GCT TCC CTG CCT TC 3'. The 4 kbp insert was XhoI/NotI digested and inserted in the pBacPAK8 vector. The resulting 9.5 kbp plasmid (pak8hisMYBBP1a) was sequenced and used to create baculoviruses according to the manufacturers protocol (Clonetech).

His-MYBBP1a was expressed by infection of Sf21 cells for 3.5 days with baculovirus. Cell pellets, were washed with PBS, resuspended and homogenised in homogenisation buffer (10 mM tris pH 7.5, 500 mM NaCl, 10 % glycerol, 0.1 % NP40, 2 mM betamercapto ethanol, 20 mM imidazole, 0.2 mM PMSF, PLB). The extract was centrifuged at 14 krpm 4 °C 30 min and filtered before loading onto a 1 ml his-TRAP column (Amersham). Bound proteins were and washed with homogenisation buffer and eluted with an imidazole gradient. Fractions were pooled, diluted and concentrated (Amicon) when required.

His-p65 was produced in a similar way using baculoviruses created with the BakPAK system and his-p300 was provided as a generous gift from Lee Kraus, Cornell University. These proteins were purified in batch using Ni2+-beads (ProBond, Invitrogen).

Regularly spaced nucleosome assembly and *in vitro* transcription

Plasmid DNA (pGL2-HIV-LTRΔtar) containing the HIV LTR with 2 NF-κB binding sites and a luciferase reporter gene was assembled into regularly spaced nucleosomes (often referred to as "chromatinisation") using drosophila S190 as described previously (Bulger and Kadonaga 1994) and drosophila histones purified and kindly provided by Dr. Andreas Ladurner. During "chromatinisation" recombinant proteins were added at the times indicated in the figure legends. *In vitro* transcription reactions were carried out as previously described [38] using HeLa nuclear extract as the source of general transcription factors [39]. Final concentrations of proteins in the reaction were 100 nM for p65, 15 nM for p300 and 50 nM for MYBBP1a. The RNA formed by the reactions was analysed by primer extension using a primer complementary to the luciferase reporter gene of the plasmid (5' GGA TAG AAT GGC GCC GGG CCT TTC 3') labelled 5' with ATPγ^{32}P. The DNA products were analysed by an 8 % urea PAGE in 1X TBE, and the signal was detected and quantified by a Phosphor Imager (Molecular Dynamics).

Results

MYBBP1a interacts with RelA/p65.

Previously we identified MYBBP1a from nuclear extracts as an interacting protein of the transcription activation domain (TAD) of RelA/p65 [20]. In order to determine whether MYBBP1a interacts directly with RelA/p65, GST pull-down experiments were performed using immobilised GST-control, GST-RelA/p65 full length or GST-RelA/p65-TAD and purified his-MYBBP1a (Figure 1A and 1B). Western blot analysis of bound material indicated that his-MYBBP1a could bind to both GST-RelA/p65 full length and TAD but not to GST alone (Figure 1C). To investigate whether contaminating DNA or RNA mediated the interaction between RelA/p65 and MYBBP1a, a similar GST pull down experiment was performed after micrococcal nuclease (MN) treatment of the proteins to remove single and double stranded DNA and RNA [36]. Coomassie staining of the experiment performed with or without MN treatment, allowed rough quantification of the binding (Figure 1D and 1E). About 20 % of the his-MYBBP1a input bound to GST-RelA/p65-TAD with or without MN treatment. More than 20 % of the his-MYBBP1a input bound to GST-RelA/p65 in the absence of MN treatment while less than 20 % bound in the presence of MN treatment. The binding was reduced at higher salt concentrations (120 mM NaCl for the washing steps). This indicates that full length RelA/p65 and the TAD domain can interact with MYBBP1a. The RelA/p65 interaction with MYBBP1a may be stabilised by DNA or RNA, while the TAD interaction with MYBBP1a is not stabilised by DNA or RNA.

Over-expression of MYBBP1a represses NF-κB dependent reporter transcription.

To analyse the effect of MYBBP1a on RelA/p65 dependent transcription, transient transfection reporter assays were performed in Jurkat T cells. Expression of RelA/p65 activated reporter expression under the control of the HIV1- Long Terminal Repeat (LTR) (black bar in Figure 2), which contains two binding sites for NF-κB but not of the same reporter where the two consensus binding sites were mutated (white bar in Figure 2). Expression of MYBBP1a repressed this activation in a concentration dependent manner (Figure 2A). Levels of the mutated HIV1-LTR reporter gene were

not significantly affected by MYBBP1a expression. In Jurkat cells, endogenous NF-κB activated by 17.5 nM PMA (phorbol 12-myristate 13-acetate) stimulation could also be repressed by expression of MYBBP1a (Figure 2B). Expression of the mutated HIV1-LTR reporter gene was slightly activated by PMA, and MYBBP1a also inhibited this activation. This indicates that the transcription activation by both over-expressed RelA/p65 and endogenous RelA/p65 activated by PMA stimulation is inhibited by expression of MYBBP1a in Jurkat cells in an NF-κB dependent manner.

Since MYBBP1a has been shown to effect the activity of other transcription factors both positively and negatively, the effect of MYBBP1a expression on p53 dependent reporter gene expression was also tested. Expression of MYBBP1a did not significantly reduce the transactivation by p53 (Figure 2C). This indicates that MYBBP1a represses only a subset of transcription factors, of which RelA/p65 is one, and p53 is not.

MYBBP1a is a cell type enriched protein with a nucleolar cellular localisation in Jurkat (T) cells.

Previously we found that higher levels of MYBBP1a bound to RelA/p65-TAD from Jurkat nuclear extracts than from HeLa or THP1 nuclear extracts. To identify whether this is due to higher levels of MYBBP1a in Jurkat nuclear extracts than in the other cell types western blot analysis was performed (Figure 3A). MYBBP1a was detected in the nuclear extracts of all the three cell types, with higher levels in Jurkat nuclear extract. No MYBBP1a was detected in the cytoplasmic extract from any cell type. Stimulation of Jurkat cells with 175 nM PMA did not alter the levels of MYBBP1a seen in nuclear, cytoplasmic or insoluble extracts (Figure 3B).

Previously, MYBBP1a was shown to be localised mainly in the nucleoli of NIH 3T3 cells [27], and also in 293T cells when expressed as a flag tagged protein [28]. To identify whether MYBBP1a is also a nucleolar protein in Jurkat T cells we performed immunoflourescence. Due to the unavailability of specific antibodies against MYBBP1a suitable for immunoflourescence, cells over-expressing myc-tagged MYBBP1a were used and stained with an anti-myc antibody. In Jurkat cells myc-MYBBP1a localises to the nucleolus (Figure 3C). These results indicate that MYBBP1a is expressed more in Jurkat (T) cells than in HeLa (epithelial) or THP1

(monocyte) cells and that MYBBP1a is found mainly in the nucleolus of Jurkat (T) cells.

MYBBP1a and p300 compete for RelA/p65-TAD binding.

Since the transcriptional cofactor p300 is known to interact with the TAD of RelA/p65 [21], we investigated whether both p300 and MYBBP1a could bind to RelA/p65-TAD at the same time. GST pull down experiments were performed using limiting amounts of GST-RelA/p65-TAD and 15 nM p300 together with varying amounts of MYBBP1a (Figure 4). Upon addition of increasing amounts of MYBBP1a (5-25 nM) we found that more MYBBP1a bound to GST-RelA/p65-TAD while less p300 bound to GST-RelA/p65-TAD (Figure 4B). Addition of 25 nM BSA had no effect on the p300 binding to GST-RelA/p65-TAD (Figure 4B). No p300 or MYBBP1a bound to GST under the same conditions (Figure 4C). This indicates that MYBBP1a and p300 cannot bind to the RelA/p65-TAD at the same time, implying that there is competition between the activator (p300) and repressor (MYBBP1a) for interaction with RelA/p65-TAD.

MYBBP1a is a co-repressor of transcription *in vitro*.

To further investigate NF-κB regulated transcription by MYBBP1a and p300, *in vitro* transcription experiments were performed on template DNA with regularly spaced nucleosomes (referred to as chromatinised templates) as shown in the scheme in Figure 5A. Nucleosomes were regularly spaced after chromatinisation in the presence or absence of RelA/p65 (Figure 5B). Without addition of transactivating factors transcription from naked (non nucleosomal) templates was detected but not from "chromatinised" templates. Addition of RelA/p65 activated transcription from the "chromatinised" templates 11.6 fold (Figure 5C, lane 5). Addition of MYBBP1a repressed this activation to 5.7 fold (Figure 5C, lane 6). p300 alone activated transcription slightly by 4.2 fold (Figure 5C, lane 3) and MYBBP1a repressed this activation to 1.4 fold (Figure 5C, lane 4). RelA/p65 and p300 activated transcription synergistically to 62 fold (Figure 5C, lane 7) and this was repressed to 13.3 fold when MYBBP1a was added to the reaction 30 minutes before p300 addition (Figure 5C, lane 8). When MYBBP1a was added to the reaction after p300, less repression was detected (figure 5C, lane 9). To exclude the possibility that MYBBP1a contained

RNAses, MYBBP1a was added at a later stage, just before the addition of HeLa nuclear extracts, but before the synthesis of RNA. MYBBP1a did not repress the transcription when added at this stage (Figure 5D). This also indicates that MYBBP1a repression of NF-κB regulated transcription occurs at a stage after transcription factor binding to chromatin, but before pre-initiation complex formation. MYBBP1a was also found to repress transcription from naked DNA templates, when added 1 hour before the nuclear extracts (Figure 5E) but not when added just before the HeLa nuclear extracts (Figure 5F), suggesting that MYBBP1a might also associate with naked DNA to form a transcriptionally repressive complex.

Discussion

MYBBP1a represses NF-κB dependent transcription.

Here, MYBBP1a was found to interact with the TAD of RelA/p65 (Figure 1) and to repress expression of an NF-κB dependent reporter gene *in vivo* driven by expression of RelA/p65 or by PMA stimulation (Figure 2). Previous work indicated that MYBBP1a might also repress the transcription factor Myb and the PPARγ co-factor PGC-1α [27, 32]. Although full length MYBBP1a had no effect on Myb reporter gene expression, the N terminal of MYBBP1a (p67) strongly repressed transactivation in CV-1 cells [27]. Transfection of both MYBBP1a and p67 repressed PPARγ/PGC-1α reporter expression in HIB1B cells. Gal4-DNA binding domain fused to MYBBP1a repressed Gal4 dependent reporter gene expression indicating MYBBP1a may have an intrinsic transcriptional repression function [32]. However MYBBP1a does not generally repress transactivation by all transcription factors as expression of MYBBP1a did not significantly effect p53 reporter gene expression (Figure 2C) and it was reported that in mouse hepatoma cells expression of MYBBP1a increased AhR dependent reporter expression [31]. This indicates that MYBBP1a may be appropriately recruited to the regulatory DNA elements by specific transcription factors in order to repress transcription. For NF-κB regulated genes this is likely to be through the direct interaction of MYBBP1a and RelA/p65-TAD (Figure 1).

Since MYBBP1a dependent repression occurred *in vivo* when using PMA activated endogenous RelA/p65 these results indicate that under some circumstances MYBBP1a may repress transcription of endogenous RelA/p65 dependent genes. Further work is needed to investigate which genes are regulated by MYBBP1a and under which cellular conditions. MYBBP1a could repress NF-κB dependent transcription in Jurkat T cells, which were found to have higher levels of MYBBP1a than THP1 monocyte or HeLa epithelial cells (Figure 3). This indicates that the MYBBP1a levels in Jurkat cells may not be saturating. Also, it is not known how high the cellular levels of MYBBP1a are in comparison to other transcriptional regulators. The only other known cell type enriched cofactor of NF-κB dependent transcription is TAF4b ($TAF_{II}105$). This TAF is part of the TFIID complex in B cells, and was found to interact with RelA/p65 and to be required for the expression of a subset of genes, through an unknown mechanism [24, 40]. B and T cells with blocked TAF4b function

had an increased apoptosis sensitivity indicating that TAF4b has an anti-apoptotic effect [41]. However, TAF4b-/- mice do not have B cell defects indicating that other TAFs are able to carry out TAF4b functions [42].

Mechanism of MYBBP1a mediated NF-κB repression.

MYBBP1a was detected to repress HIV1-LTR driven transcription *in vitro* using chromatinised templates as the substrate (Figure 5). In the experiments RelA/p65 was pre-bound to the DNA. MYBBP1a could repress transcription when added to the chromatin RelA/p65 complex before addition of p300, but not when added at a later stage (Figure 5). This indicates that MYBBP1a can repress transcription at a step after NF-κB binds to DNA, but does not directly repress assembly of the pre-initiation complex containing general transcription factors and pol II. This is the same stage of transcription at which p300 can activate transcription by acetylation events [43]. p300 mediates acetylation of core histone tails and other transcription regulatory proteins at specific promoters by interaction with DNA binding transcription factors. We do not exclude the possibility that MYBBP1a can also repress other steps in the NF-κB activation pathway. Here we show that MYBBP1a can interact with the transcriptional activation domain (TAD) of RelA/p65 (Figure 1) and competes for this domain with the transcriptional co-activator p300 (Figure 4). The interaction between MYBBP1a and TAD could inhibit the transcriptional potential of the TAD by exclusion of p300. Therefore, these results support the hypothesis that MYBBP1a might function as a co-repressor of transcription by altering the accessibility of the promoter by competition with p300 histone acetyltransferase activity. It is also possible that MYBBP1a could be part of or recruit a transcriptional repressor complex. Evidence for such an histone deacetylase containing repressor complex (HDAC) is that the repression of reporter gene expression by Gal4-MYBBP1a is relieved upon HDAC inhibitor (trichostatin A) treatment [32].

In vitro MYBBP1a also repressed transcription from naked templates when added one hour before the nuclear extracts. This indicates that MYBBP1a repressed transcription of naked DNA template possibly by associating with the DNA template, causing a transcriptionally repressive state. Further experiments are required to determine the characteristics of this slow association and whether it is relevant for transcription repression in the cellular context. An association with MYBBP1a and

chromatinised templates is less likely than with naked DNA due to the presence of nucleosomes and other chromatin proteins. Therefore, the repression of RelA/p65 and p300 activated transcription on chromatinised templates may be mainly due to the MYBBP1a competition with p300.

Regulation of MYBBP1a repression.

In this work we show that MYBBP1a and p300 compete for RelA/p65-TAD interaction (Figure 4). The equilibrium between these proteins bound to RelA/p65 is likely to determine the transcriptional potential of RelA/p65 and could be carefully regulated at specific promoters to create the appropriate pattern of gene expression. This equilibrium could be regulated by the levels of p300 and MYBBP1a present in different cell types and also by signalling events. p300 was previously shown to be limiting in cells [44, 45]. Although MYBBP1a mRNA transcripts were found in most tissues and cell lines tested [27] [46], it cannot be assumed that MYBBP1a protein is present since the degradation of MYBBP1a to the stable p67 degradation product was detected only in a subset of cell types [27]. The expression levels of p67 in various cell types and the function of p67 in NF-κB dependent transcription are unknown. Data from micro-array analysis on the Human Gene Atlas website (http://symatlas.gnf.org/SymAtlas/) [47], shows that mRNA expression was three times above median for many cells of the immune system, indicating that these cells might have elevated levels of MYBBP1a and/or p67. Here we show that MYBBP1a protein level is higher in Jurkat T cells than THP1 monocytes or HeLa epithelial cells (Figure 3). This indicates that the level of MYBBP1a can be different in specific cells types, and may be particularly high in T cells, implying it may have a special role in T cell signalling. Proteomic approaches would be ideal to determine the difference in levels of MYBBP1a in various normal tissues and cells.

Signalling events could also lead to specific patterns of post-translational modification of RelA/p65, p300 and MYBBP1a, regulating the interaction between them. This would be analogous to co-activator/co-repressor exchange during nuclear hormone receptor activation by ligand binding. It has been shown that phosphorylation of RelA/p65-TAD by calcium/calmodulin dependent kinase IV increased the interaction between RelA/p65-TAD and CBP (a close p300 homologue) whilst reducing the affinity between RelA/p65-TAD and the co-repressor complex

SMRT [48]. In the interaction studies presented here non-modified RelA/p65-TAD was used which may favour the interaction with specific proteins such as transcriptional repressor proteins.

Here we show that expressed myc-MYBBP1a localised mainly to the nucleolus of human Jurkat T cells (Figure 3). Endogenous MYBBP1a is also present mainly in the nucleolus of mouse NIH 3T3 fibroblasts [27] and human HeLa cells [49]. MYBBP1a was previously shown to have nuclear import and export signals and to move between the nucleus and cytoplasm [28]. As a flag tagged exogenously expressed construct it moved from the nucleoli in one nucleus to another nucleoli in a different cell in a heterokaryon (fused cell) experiment [28]. It is not known whether the nucleolar/nuclear/cytoplasmic shuttling of MYBBP1a is regulated within the cell, although preliminary data suggest that upon PMA stimulation there may be some nucleolar to cytoplasmic shuttling of over-expressed myc-MYBBP1a (H. Owen and M. Hottiger, unpublished).

Since RelA/p65 is not known to have a transcription function in the nucleolus perhaps the small amount of MYBBP1a found diffuse in the nucleus is able to regulate RelA/p65 and other pol II transcription factors. It is also possible that MYBBP1a could sequester RelA/p65 in the nucleoli, thereby inhibiting RelA/p65 dependent transcription. RelA/p65 was previously shown to be sequestered in the nucleolus upon cell stimulation with the pro-apoptotic signals aspirin, UV-C and serum deprivation [50]. This sequestration led to an inhibition of NF-κB dependent reporter transcription.

To conclude, we find that MYBBP1a is a novel co-repressor of NF-κB dependent transcription. MYBBP1a repressed PMA activated endogenous NF-κB as well as RelA/p65 over-expressed mediated induction of NF-κB dependent reporter expression in transient transfection assays. MYBBP1a also inhibited RelA/p65 and p300 activated transcription in reactions performed with chromatin templates *in vitro*. MYBBP1a repression was seen at a stage after RelA/p65 binding to DNA, but before addition of general transcription factors from a nuclear extract source. MYBBP1a interacts with RelA/p65-TAD in a competitive manner with the transcriptional co-activator p300. This indicates that MYBBP1a might function as a co-repressor of transcription at a stage before PIC formation by competing with p300.

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Figure Legends

Figure 1. MYBBP1a interacts with the transcription factor RelA/p65 via the transcription activation domain.

(A) and (B) Proteins used for the GST pull down experiments analysed by SDS-PAGE and coomassie staining. Arrow heads indicate the positions of GST, GST-RelA/p65, GST-RelA/p65-TAD (A) and his-MYBBP1a (B).

(C) GST pull down experiment was performed using the proteins shown in A and B and binding and washing conditions of 50 mM NaCl or 80 mM NaCl. Bound material was analysed by western blot using an anti-his antibody to detect his-MYBBP1a. 5% and 20% of input his-MYBBP1a were included on the western blot. His-MYBBP1a interacts with GST-RelA/p65 and GST-RelA/p65-TAD but not GST.

D) GST pull down experiments were performed using binding/washing conditions of 97/80 mM NaCl or 135/120 mM NaCl, and bound material was visualised by coomassie staining. Positions of GST, GST-RelA/p65, GST-RelA/p65-TAD, his-MYBBP1a are indicated with arrow heads. An Asterix shows the position of MYBBP1a binding at 97 mM NaCl.

(E) GST pull down experiments were performed as in D, with an additional micorococcal nuclease digestion step before the interaction study, to digest contaminating single and double stranded oligonucleotides.

Figure 2. Expression of MYBBP1a represses NF-κB dependent reporter gene expression.

(A) Graph to show the fold activation of HIV1-LTR reporter gene expression in Jurkat cells when induced by RelA/p65. Cells were transiently transfected with pGL2-HIV1-LTR wild type (black bar) or pGL2-HIV1-LTR-ΔκBMUT (white bar) reporter, and the indicated expression plasmids. Luciferase levels were normalised against total protein level. Samples were in duplicate and the mean used. The graph shows the results for the mean of three independent experiments, where the bars are the standard deviation of the three experiments.

(B) Graph to show the fold activation of HIV1-LTR reporter gene expression in Jurkat cells when induced by 17.5 nM PMA. Cells were transfected and luciferase levels were measured as in A. The graph shows the results for the mean of three

independent experiments, where the bars are the standard deviation of the three experiments.

(C) Graph to show the fold activation of BS2 reporter gene expression in Jurkat cells when induced by p53. Cells were transfected and luciferase levels were measured as in A. Reporters used were BS2-wild type (black bars) and BS2-mutant (white bars). The graph shows the results for the mean of three independent experiments, where the bars are the standard deviation of the three experiments.

Figure 3. Higher levels of MYBBP1a are present in Jurkat (T) cells than in THP1 (monocyte) or HeLa (epithelial) cells and myc-tagged MYBBP1a localises to the nucleolus in Jurkat cells.

(A) Western blot analysis of nuclear and cytoplasmic extract from Jurkat (T), THP1 (monocyte) or HeLa (epithelial) cells. Antibodies against tubulin, PCNA, PARP and RelA/p65 were used as loading controls. PARP is a nuclear protein, while in non-stimulated cells RelA/p65 is cytoplasmic.

(B) Western blot analysis of nuclear, cytoplasmic and insoluble extracts from Jurkat (T) cells with and without 175 nM PMA stimulation for 1 hr.

(C) MYBBP1a is mainly nucleolar in Jurkat T cells. Immunoflourescence of Jurkat cells transfected with a myc-MYBBP1a expression vector. Cells were stained with anti-myc (left) and DAPI (middle). Light fields were also visualised to distinguish nucleoli (right). Two representative transfected cells are shown, with bright green staining. Faint dark green staining is the background signal.

Figure 4. MYBBP1a competes with p300 for binding to RelA/p65-TAD.

(A) Proteins used for the GST pull down experiments analysed by SDS-PAGE and coomassie staining were GST, GST-RelA/p65-TAD, BSA, his-p300 and his-MYBBP1a.

(B) GST pull down experiment performed with GST-RelA/p65-TAD and salt conditions of 80 mM NaCl for binding and washing. Constant amount of his-300 was used whilst MYBBP1a amount was increased as indicated. BSA was used as a negative control. Bound material was analysed by western blot using antibodies against p300 or MYBBP1a.

(C) GST pull down experiment performed with GST control and salt conditions of 80 mM NaCl for binding and washing. Bound material was analysed by western blot using antibodies against p300 or MYBBP1a.

Figure 5. MYBBP1a represses transcription *in vitro* of HIV1-LTR reporter DNA assembled into regularly spaced nucleosomes.

(A) Transcription scheme. S190 extract is incubated with drosophila core histones for 30 min at RT. RelA/p65 or elution buffer is incubated with pGL2-HIV1-LTR-WT for 20 min on ice. RelA/p65-DNA is incubated with the S190-histones, plus an MgATP generation mix at 27 °C for 3 h 30 min. Transcription reactions are then performed as described in the scheme and in the Experimental section. HNE stands for HeLa nuclear extracts. PIC stands for Pre-initiation complex formation. RT stands for room temperature.

(B) Samples of the chromatinisation reactions carried out in the presence and absence of RelA/p65 are analysed by micrococcal nuclease digestion to ensure efficient deposition of regularly spaced nucleosomes.

(C) Transcription reactions were carried out with the combination of proteins indicated. MYBBP1a was added at A or B as shown in the transcription scheme in Figure 5A. Transcription reactions were analysed by primer extension with a ^{32}P labelled primer and 8% urea PAGE. The experiment was performed three times and an example radiograph is shown. Most samples were in duplicate and the mean taken. The fold activation for each experiment was calculated by setting the sample without proteins added (lane 1) as 1. The mean fold activation (F.A.) and the standard deviation (S.D.) for the three experiments was calculated and is shown.

(D) Transcription reactions were carried out as in C, although MYBBP1a (M) was added just before the HeLa nuclear extracts at time point C in the transcription scheme in Figure 5A.

(E) Naked transcription reactions (on non-chromatinised DNA) were carried out. MYBBP1a (M) was added 1 h before the HeLa nuclear extracts at time point A in the transcription scheme in Figure 5A.

(F) Naked transcription reactions were carried out. MYBBP1a (M) was added just before the HeLa nuclear extracts at time point C in the transcription scheme in Figure 5A.

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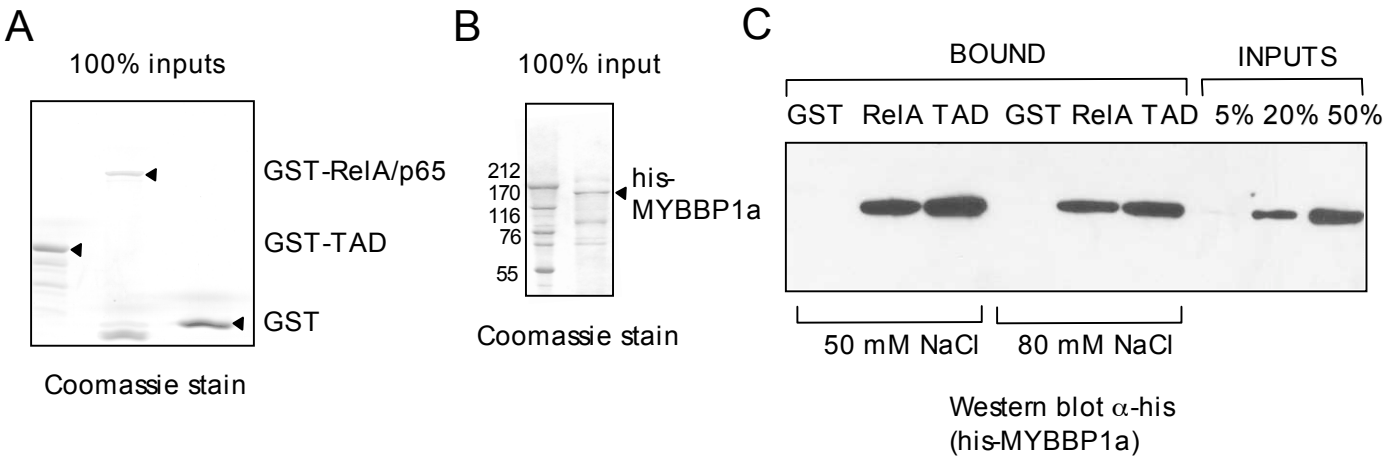
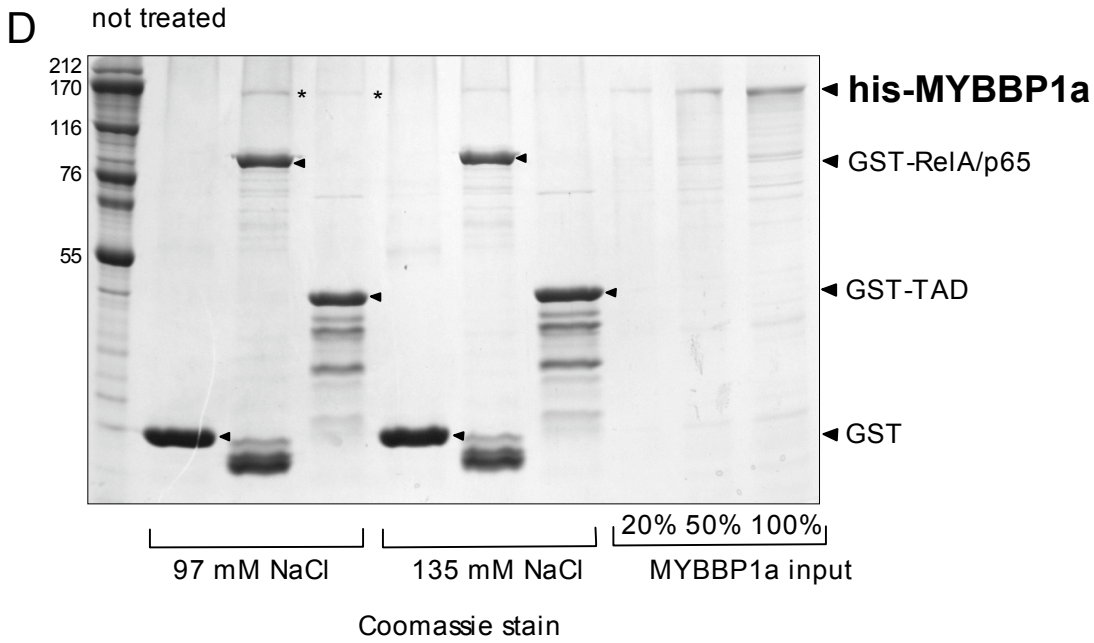
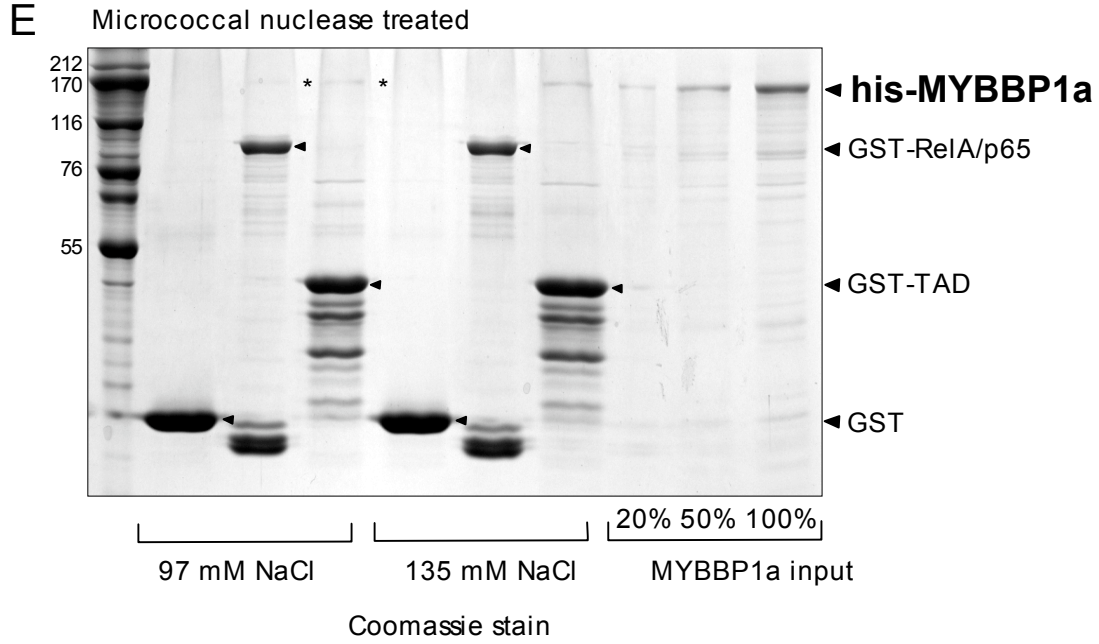
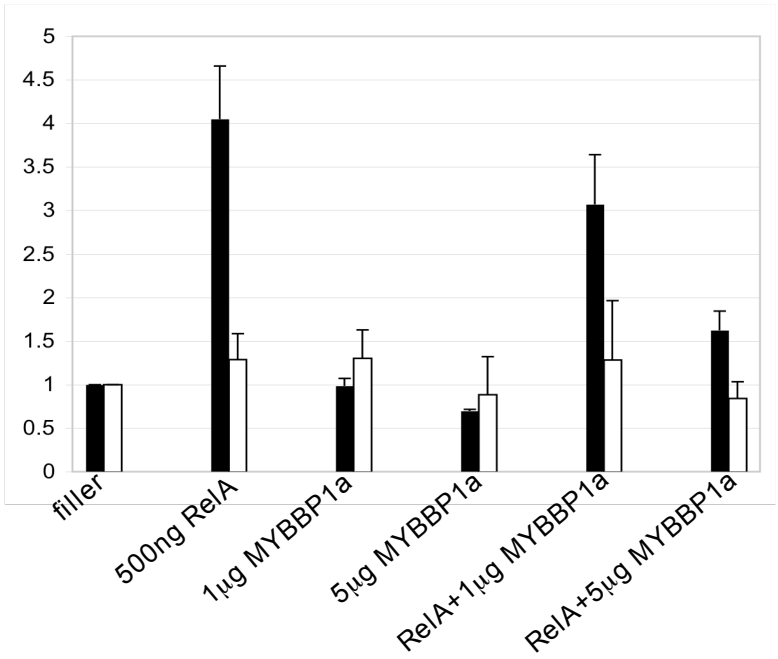

A
100% inputs
GST-RelA/p65
GST-TAD
GST
Coomassie stain
B
100% input
212
170
116
76
55
his-MYBBP1a
Coomassie stain
C
BOUND
INPUTS
GST RelA TAD GST RelA TAD 5% 20% 50%
50 mM NaCl
80 mM NaCl
Western blot α-his
(his-MYBBP1a)

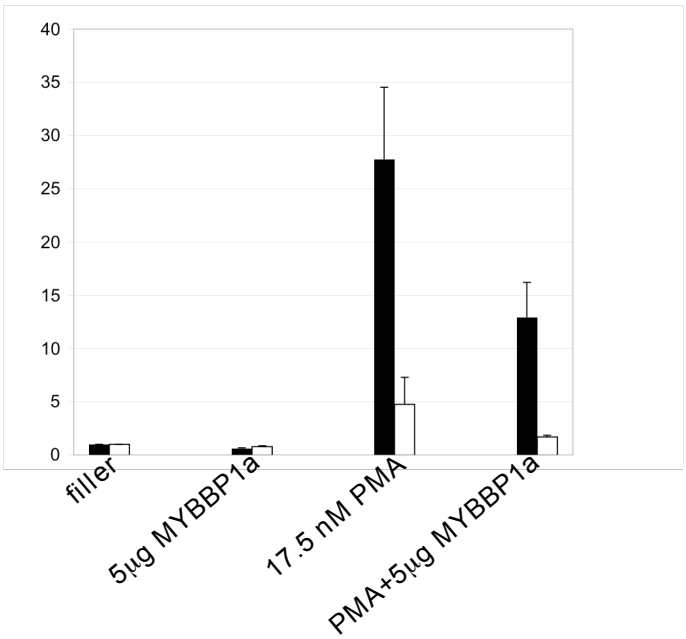

D
not treated
212
170
116
76
55
his-MYBBP1a
GST-RelA/p65
GST-TAD
GST
20% 50% 100%
97 mM NaCl
135 mM NaCl
MYBBP1a input
Coomassie stain


E
Micrococcal nuclease treated
212
170
116
76
55
his-MYBBP1a
GST-RelA/p65
GST-TAD
GST
20% 50% 100%
97 mM NaCl
135 mM NaCl
MYBBP1a input
Coomassie stain

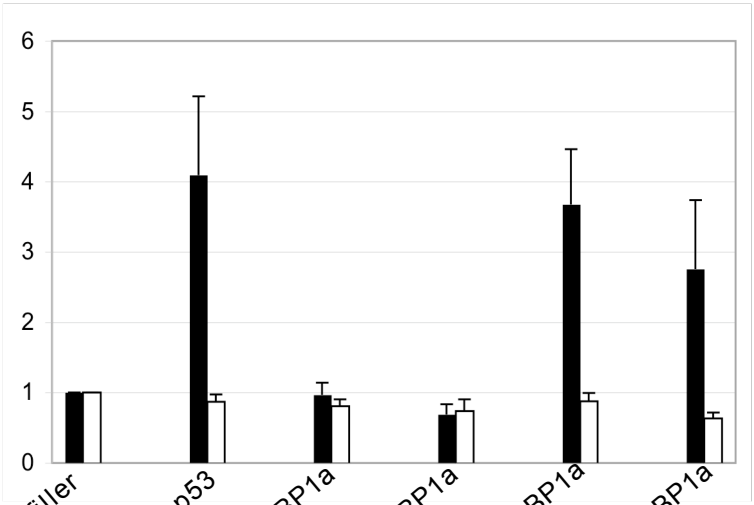
A


5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
filler
500ng RelA
1μg MYBBP1a
5μg MYBBP1a
RelA+1μg MYBBP1a
RelA+5μg MYBBP1a

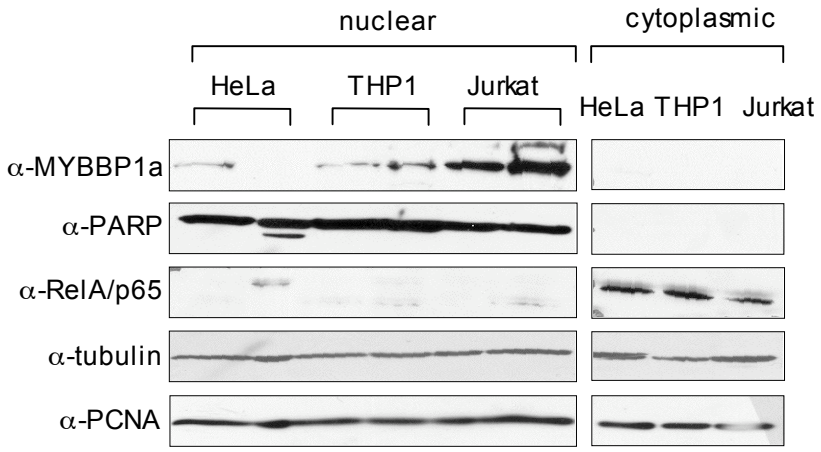
B


40
35
30
25
20
15
10
5
0
filler
5μg MYBBP1a
17.5 nM PMA
PMA+5μg MYBBP1a

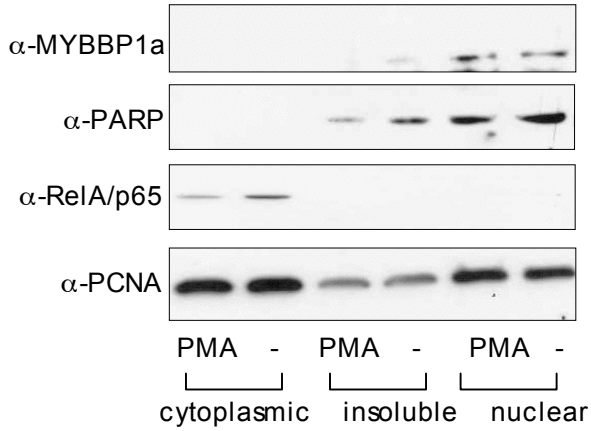
C


6
5
4
3
2
1
0
filler
500ng p53
1μg MYBBP1a
5μg MYBBP1a
p53+1μg MYBBP1a
p53+5μg MYBBP1a

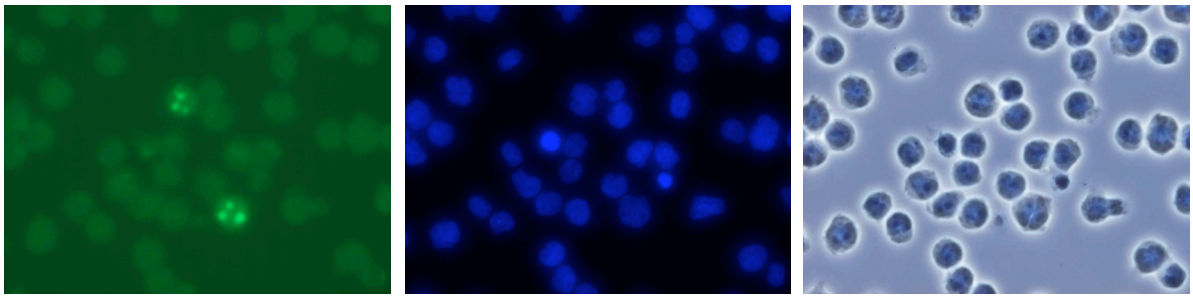
A



B

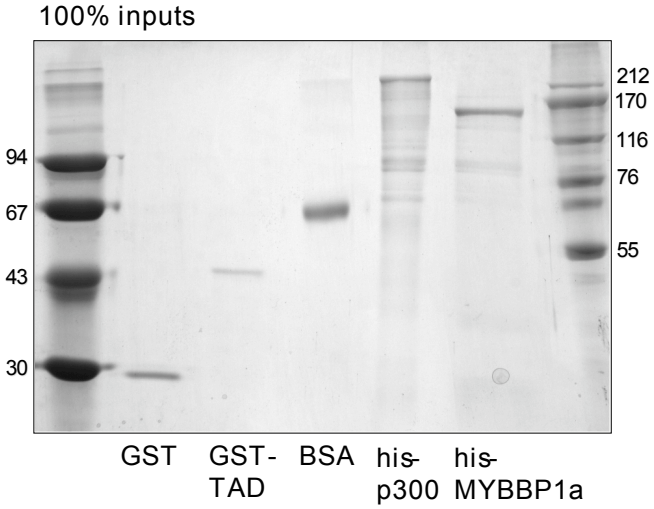


C



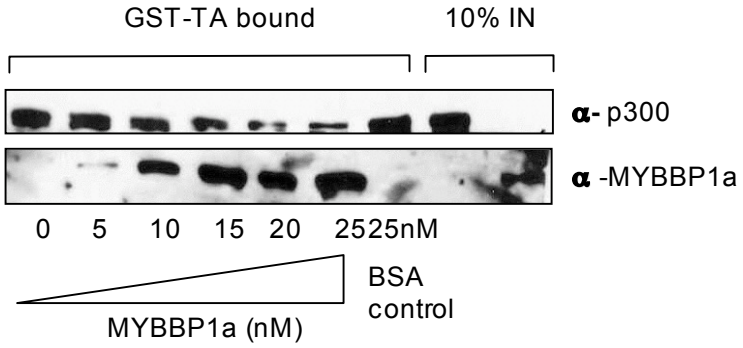
myc-MYBBP1a (anti-myc) DAPI light

A



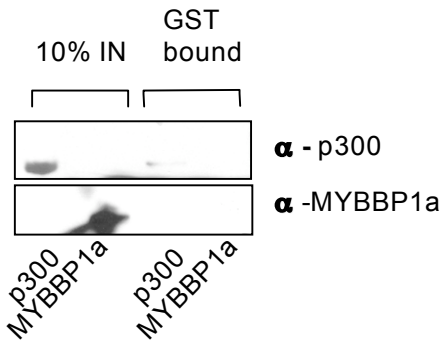
Coomassie stain

B



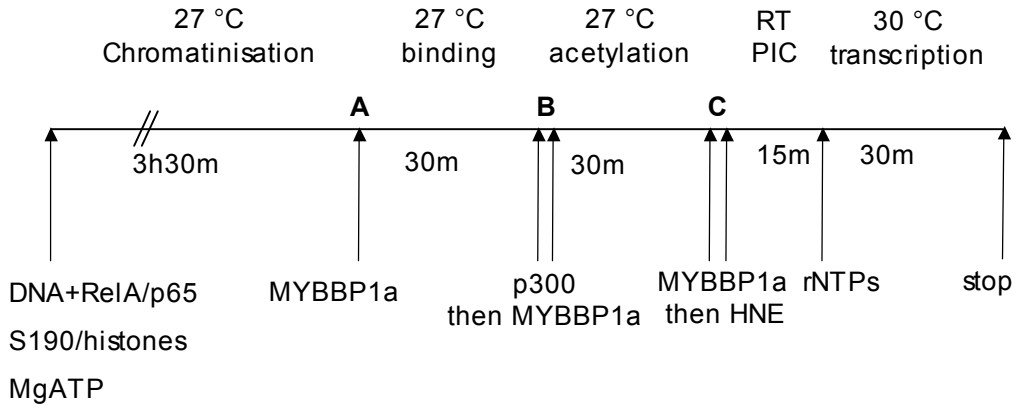
Western Blot

C

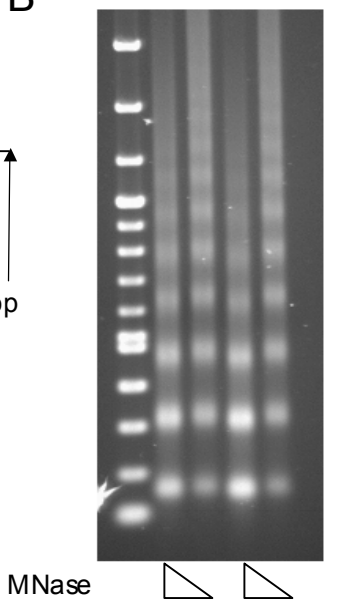


Western Blot

A

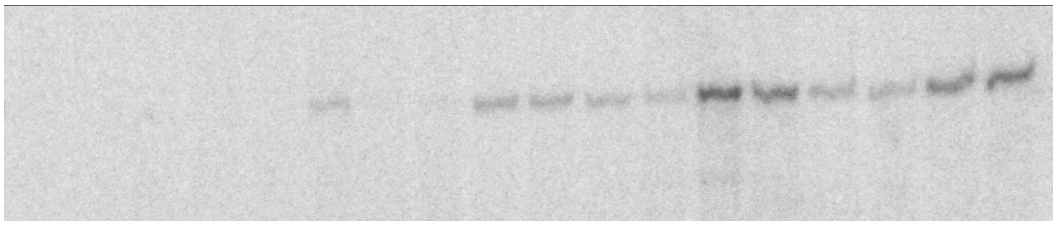


B



C

RelA/p65	-	-	-	-	+	+	+	+	+
p300	-	-	+	+	-	-	+	+	+
MYBBP1a	-	**at A**	-	**at A**	-	**at A**	-	**at A**	**at B**



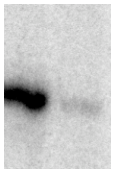
lane	1	2	3	4	5	6	7	8	9
F.A.	1	1.2	4.2	1.4	11.6	5.7	62.0	13.3	28.8
S.D.	0	0.5	0.5	0.7	3.3	3.1	17.8	5.5	13.9

D

RelA/p65	+	+
p300	+	+
MYBBP1a	-	**at C**

E

RelA/p65	-	-
p300	-	-
MYBBP1a	-	**at A**



naked

F

RelA/p65	-	-
p300	-	-
MYBBP1a	-	**at C**

naked

MODULATION OF GENE-EXPRESSION BY p300-MEDIATED ACETYLATION OF RELA/p65

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Running title: Acetylation of NF-κB by p300/CBP

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Nuclear factor kappaB (NF-κB) has an important role in the transcriptional regulation of genes involved in immunity and cell survival. We show here that RelA/p65 is acetylated by p300. Acetylation sites were mapped *in vitro* to lysine K310, K314 and K315. Genetic complementation of RelA/p65 -/- cells with wild type and non acetylatable mutants of RelA/p65 (K310R, K314R and K315R) revealed that neither shuttling nor DNA binding of RelA/p65 was affected by acetylation. Microarray analysis of these cells treated with TNFα identified a specific set of genes that was regulated differently by wild type or acetylation deficient RelA/p65. Specific genes were identified to be either stimulated or repressed by the acetylation deficient mutant when compared to RelA/p65 wild type. These results support the hypothesis that p300-mediated acetylation of RelA/p65 at defined sites is important to regulate the specificity of gene expression.

The inducible transcription factor family nuclear factor κB (NF-κB) consists of dimeric proteins involved in many diverse processes such as immune and stress responses and even the opposing processes of proliferation and apoptosis (1-3). NF-κB is induced in almost all cell types by different extracellular stimuli causing the activation of an enormous array of target genes (4). Thus it is not surprising that the specificity of NF-κB responses is very important for the fate of a cell. It has been shown that abnormal NF-κB activity, that is not always associated with genetic alterations, plays a role in different inflammatory diseases and cancer (5,6).

NF-κB specificity is regulated at different levels in the cell (7). One level of regulation is the selective activation of different NF-κB complexes after induction by diverse stimuli. In mammals there are five family members, c-Rel, RelB, p65 (RelA), p105/p50 (NF-κB1) and p100/p52 (NF-κB2) that can form a range of homo- and heterodimers (8). After regulated IκB (inhibitor of NF-κB)-dependent NF-κB translocation to the nucleus these dimers bind with variable affinities to consensus NF-κB binding sites in the promoter and enhancer regions of their target genes, often cooperatively with other transcription factors (e.g., at the IFNβ promoter (9)). This integrates other signal transduction pathways with the NF-κB pathway giving additional levels of specificity and regulation to the transcriptional control of responsive genes. The interaction with cell type specific co-factor proteins has been shown to influence the transcriptional potential of NF-κB (10). One of the co-factors of NF-κB is the co-activator p300 and its homolog CBP (CREB-binding protein). They have been shown to interact with the RelA/p65 and the p50 subunit (9,11-13) serving as molecular bridges between NF-κB and the transcription machinery. They also contain intrinsic histone acetyltransferase activity catalyzing the acetylation of lysine residues in histones and non-histone proteins (14,15). A growing number of transcription factors are acetylated and regulated by p300/CBP including p53 (16), GATA-1 (17), E2F-1 (18,19) and YY1 (20). Post-translational acetylation influences different properties of

these transcription factors such as DNA binding, protein-protein interactions, protein stability and transcriptional potential (reviewed in (21)).

NF-κB is subjected to a variety of post-translational modifications (e.g., phosphorylation (22), ubiquitination (23) or prolyl-isomerisation (24)) that modulate NF-κB activity. Phosphorylation of the RelA/p65 subunit by the PKAc, MSK1 and PKCζ kinases enhances its interaction with the co-activator p300/CBP and stimulates the NF-κB transcriptional activity (25-27). In contrast, ubiquitination of RelA/p65 on the promoter specifically terminates the NF-κB response (23).

It has recently been shown that RelA/p65 and p50 are reversibly acetylated by p300 and PCAF ((28-30)). Chen *et al*. identified lysine residues (K) 218, 221 and 310 of RelA/p65 as acceptor sites for p300 acetylation. They reported that acetylated lysine 221 enhanced DNA binding activity of NF-κB *in vitro* and abolished the interaction with IκBα leading to a prolonged NF-κB response in the nucleus. The acetylation at lysine residue 310 was required for full transcriptional activity of RelA/p65 (31). Kiernan *et al*. identified lysine 122 and 123 in RelA/p65 as acetylation sites modified by both p300 and P/CAF. In contrast to K218, K221 and K310, acetylation of K122 and K123 decreased the DNA binding of RelA/p65 facilitating the removal of RelA/p65 from the DNA and the export from the nucleus by IκBα resulting in a faster termination of the NF-κB response (29). These data question the precise role of RelA/p65 post-translational acetylation in NF-κB dependent gene regulation *in vivo*.

The aim of this study was to identify the role of RelA/p65 acetylation *in vivo*. We found that p300 efficiently acetylated RelA/p65 *in vitro* at lysines 310, 314 and 315. We generated acetylation deficient lysine to arginine substitution mutants of RelA/p65 and stably complemented RelA/p65-/- cells with these mutants. The nuclear-cytosolic shuttling and the DNA binding of the acetylation deficient mutants were similar to that of wild type RelA/p65. However, whole genome microarray analysis indicated that the expression of specific genes was affected by the K/R mutation. Our results imply that although general transcriptional activity of RelA/p65 was not affected by acetylation at lysine 310, 314 and 315, the expression of a specific set of genes was modulated. Thus, acetylation can serve as a molecular mechanism to promote specificity of NF-κB-dependent gene expression.

Experimental procedure

Plasmids - hGCN5L, mP/CAF and hTip60 were cloned into pFastBacHTb vector in frame with a N-terminal 6xHis-tag. pph-CMV-Km-RelA/p65 wild type was previously described in (13). pph-CMV-Km-RelA/p65K310R, pph-CMV-Km-RelA/p65K314/K315R and pph-CMV-Km-RelA/p65K310R/K314/K315R were generated by site-directed mutagenesis according to the QuickChange protocol (Stratagene) using the following oligonucleotides: K310R: 5'CGTAAAAGGACATACGAGACCTTCA**G**GAGCATCATGAAGAAGAGTCC3' and 5'GGACTCTTCTTCATGATGCTC**C**TGAAGGTCTCGTATGTCCTTTTACG3', K314/315R:5'CCTTCAGGAGCATCATG**CG**GA**G**GAGTCCTTTCAGCGGACCC3' and 5'GGGTCCGCTGAAAGGACTC**C**TC**CG**CATGATGCTCCTGAAGG3' (bold letters represent K/R mutation). All introduced mutations were confirmed by sequencing.

Reagents and Antibodies - Mouse TNFα, Trichostatin A (TSA), Nicotinamide (NAM), acetyl-Coenzyme A and calf thymus core histones (H7755) were purchased from Sigma. ^{14}C-labeled acetyl Coenzyme A (MC269) was obtained from Moravek Biochemicals. Most of antibodies were purchased from Santa Cruz Biotechnology: anti-RelA/p65 (C-20, sc-372), anti-p300 (C-20, sc-585) and anti-α-tubulin (TU-02, sc-8035). The anti-p50 antibody was a generous gift from N. Rice (National Cancer Institute, Frederick, MD). Anti-myc 9E10 antibody was purified from hybridoma cells according to the standard protocol. The tetra-his mouse monoclonal IgG1 antibody was from Qiagen. The specific antibody against acetylated lysine 310 in RelA/p65 was generated in collaboration with Abcam.

Tissue culture, cell transfections - Complemented RelA/p65-/- NIH 3T3 mouse embryonic fibroblasts (MEFs) and HEK 293T cells were maintained in DMEM supplemented with 10% FCS, 100 Units/ml penicillin/streptomycin and non-essential amino acids (GIBCO). Cells were transfected using calcium phosphate precipitation method.

Generation and purification of baculovirus expressed proteins - All recombinant proteins were expressed in Sf21 cells using the Bac-To-

Bac (GIBCO) or BacPAK (Clontech) system. Recombinant His-tagged proteins were purified over Ni^{2+}-beads (ProBond, Invitrogen).

In vitro acetylation assay - 1μg of recombinant human wild type or mutant RelA/p65 was incubated with 0.5-1μg recombinant p300 or CBP or equimolar amount of hGCN5L, mP/CAF or hTip60 in HAT buffer (50mM Tris-HCl pH 8.0, 100mM NaCl, 10% glycerol, 1mM DTT, 1mM PMSF, 1μg/ml pepstatin, bestatin, leupeptin, 1mM Sodium butyrate) supplemented with 1.5nmol [^{14}C]-Acetyl CoA for 45 min at 30°C. The reactions were stopped by adding 10x Laemmli-buffer. The proteins were resolved on SDS-PAGE and visualized by Coomassie brilliant blue or SyproRuby staining. The gel was immersed in 1M Sodium salicylate for 20 min at RT. After drying, the gel was exposed to X-ray films (Contatyp) at -80°C.

In vitro transcription assay - Plasmid DNA (pGL2-HIV-LTRΔtar) containing the HIV LTR with 2 NF-κB binding sites and a luciferase reporter gene was assembled into regularly spaced nucleosomes (often referred to as chromatinisation) using drosophila S190 extract as described previously (32) and human histones purified from HeLa cells as described in Current Protocols in Molecular Biology (21.5.1). During chromatinisation recombinant proteins were added at the times indicated in the figure legends. *In vitro* transcription reactions were carried out as previously described (33) using HeLa nuclear extract as the source of general transcription factors (34). Briefly, the RNA formed by the reactions was analyzed by primer extension using a primer complementary to the luciferase reporter gene of the plasmid (5'-GGA TAG AAT GGC GCC GGG CCT TTC- 3') labeled 5' with ATPγ^{32}P. The DNA products were analyzed by an 8 % urea PAGE in 1X TBE, and the signal was detected and quantified by a Phosphor Imager (Molecular Dynamics). All reactions were performed in duplicate and each experiment was repeated a minimal of three times.

Electrophoretic mobility shift assay (EMSA) - Binding reactions were done in a total volume of 20 μl containing 10mM Tris-HCl (pH 7.5), 50mM NaCl, 1mM DTT, 2mM PMSF, 0.25μg poly dI-dC, 7% (v/v) Ficoll/glycerol, 0.25 pmol HIV-LTR oligonucleotide containing 2 κB binding sites labeled 5' with ATPγ^{32}P and 7 μg of nuclear extract. The reaction was incubated for 20 minutes on ice and then resolved using a 5% polyacrylamide gel in 0.5x TBE. The gel was run at 150V for 3 hours, dried and subjected to autoradiography. When supershifts were performed the binding reaction was pre-incubated with the indicated antibody for 20 min on ice before the labeled oligonucleotide was added.

Immunohistochemistry - Cells were plated at the density of 45 000 cells per chamber on poly-L-lysine (Sigma) coated chamber slides (LAB-TEK) and incubated over night at 37°C and 5% CO_2. Next day the cells were treated with 30μg/ml of TNFα for the indicated time. The cells were fixed in 4% paraformaldehyde and then permeabilized with 0.2% Triton-X-100/PBS. After blocking for 1 hr in 2% BSA/0.1% Triton-X-100/PBS slides were incubated with anti-RelA/p65 C-20 antibody (1:300 dilution) followed by anti-rabbit Cy3 (Jackson Immunology) antibody (1:250 dilution). The samples were washed and Vectashield mounting solution (Vector laboratories) was applied to prevent bleaching. Cells were visualized using an Olympus T50 microscope.

GST-pull down experiments - GST, GST-RelA/p65wt and GST-RelA/p65KTR proteins were immobilized on glutathione beads (Amersham Pharmacia) and incubated with purified his-p300 in binding buffer (20mM Hepes pH7.5, 60mM NaCl, 10% glycerol, 1.5mM $MgCl_2$, 1mM DTT, 1mM PMSF and 1μg/ml pepstatin, bestatin, leupeptin) for 2 hrs at 4°C rolling. Glutathione beads were washed with binding buffer. Proteins were boiled, resolved on SDS-PAGE and subjected to Western blot analysis using anti-his antibody (Qiagen).

Nuclear extract preparation and immunoprecipitation - Nuclear extracts were prepared as previously described in (35). 200 μg of TNFα (30ng/ml) and HDAC inhibitor (TSA (2μM), NAM (5mM)) treated nuclear extracts of the complemented cells were incubated with 2μg of anti-RelA/p65 C-20 antibody for 2.5 hrs at 4°C rolling in binding buffer (20mM Hepes pH7.9, 80mM NaCl, 2.5mM $MgCl_2$, 0.05% NP-40, 1mM PMSF, 1μg/ml pepstatin, bestatin, leupeptin). After incubation with protein G-sepharose beads (Amersham Pharmacia) for another hour, the immunocomplexes were extensively washed in washing buffer (20mM Hepes pH7.9, 100mM NaCl, 2.5mM $MgCl_2$, 0.05% NP-40, 1mM PMSF, 1μg/ml pepstatin,

bestatin, leupeptin). Proteins were boiled, resolved on SDS-PAGE and analyzed by western blot using the anti-RelA/p65ac310. Membranes were reprobed with anti-RelA/p65 antibody.

MS/MS - *In vitro* acetylated RelA/p65 was resolved on SDS-PAGE, fixed and stained with Coomassie brilliant blue. The corresponding protein band was then excised and washed twice with 50% acetonitrile. After tryptic digestion the protein sequence analysis was performed at the Harvard Microchemistry Facility by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Finnigan LCQ DECA XP quadrupole ion trap mass spectrometer.

Lentiviral complementation of RelA/p65-/- MEFs - Virus production and transduction of RelA/p65-/- MEFs were performed as described in (36). Briefly, 293T HEK cells were transfected with 3.5 μg of the envelope plasmid, 6.5 μg of packaging plasmid, and 10 μg of pTV-myc-RelA/p65 wild type, RelA/p65 K/R or the control pTV vector. After 24 hrs the viral supernatant was harvested and used to infect RelA/p65-/-MEFs. 36 hrs post infection cells were split into selective medium containing 2.5 μg/ml Blasticidin (Sigma). Expression of recombinant proteins in the complemented cells was screened by western blot analysis. Pools of cells were used for further analysis.

RNA preparation - Total RNA was isolated three times from TNFα treated lysates from complemented RelA/p65$^{-/-}$ MEFs with the 'Total RNA isolation kit' (Agilent Technologies). RNA quality was checked with the RNA 6000 Nano kit using the Bioanalyzer 2001 (Agilent Technologies). Purified RNAs were converted into double-stranded cDNA and transcribed into Cy3-/Cy5 (PerkinElmer/NEN Life Science) - labeled cRNA using the 'Low RNA Input Linear Amp Kit' (Agilent Technologies). cRNA from wild type RelA/p65 cells was Cy5-labeld while the RelA/p65KTR cRNA was Cy3-labeled. The purification of the labeled cRNAs was done with the RNeasy kit (Qiagen). Dye incorporation was measured on the ND-1000 Spectrophotometer (NanoDrop Technologies)

Gene expression profiling - Gene expression profiling was performed in the Functional Genomics Center Zurich using the Agilent Microarray system (Agilent Technologies). 1μg of fragmented Cy5-labeled wild type and 1 μg of Cy3-labeled mutant cRNA were co-hybridised on the Whole Mouse genome 60mer-oligo array (G4122A, Agilent Technologies) according to the manufacturers protocol. The microarray analysis was performed in triplicates. The slides were scanned using the Agilent DNA microarray scanner and the scans were quantified with the Agilent Feature Extraction software.

Data analysis - Data analysis was done with the GeneSpring software (Silicon Genetics). A significance value of $p \leq 0.01$ was applied. Genes with a fold induction of more then 1.6 or less then 0.625 between mutant and wild type cell line were considered to be significantly up- or down regulated. Screening for the NF-κB consensus sequence GGGRNNYYCC in the upstream regions of the respective genes was also performed with the GeneSpring software.

Quantitative real time RT-PCR - Total RNA from untreated or 45 min TNFα treated cell lines (RelA/p65wt and RelA/p65KTR) was reverse transcribed using the high capacity cDNA Archive kit according to manufacturers protocol (ABI). Real time PCR was performed using mouse specific TaqMan probes (Gene expression assays, ABI) for Ccl-7, Fhl-1 and Mip-2. The 18SrRNA probe was used to normalize for differences in RNA input. The Rotor-Gene3000A real time PCR machine (Corbett) was used and the included data analysis software Rotor-Gene6 was applied. The figures show the results of three independent experiments.

RESULTS

RelA/p65 is acetylated in vitro by p300 or CBP.

Since RelA/p65 was reported to interact directly with several histone acetyltransferases (HAT), we investigated whether RelA/p65 could serve as a substrate in an *in vitro* acetylation assay. We compared the ability of different HATs to acetylate RelA/p65 *in vitro*. Full length RelA/p65 was incubated with recombinant p300, CBP, GCN5L, P/CAF or Tip60 (all proteins expressed and purified from insect cells, Fig. 1*A*) in the presence of radioactively labeled acetyl-coenzyme A (acetyl-CoA) as a donor of the acetyl group (Fig. 1*B*). All tested HATs, except for Tip60, acetylated RelA/p65 *in vitro* (Fig. 1*B*). Calf thymus core histones were used as a positive control of acetylation (Fig. 1C). p300 and CBP were the most potent HATs for RelA/p65 in our system. This prompted us to focus on p300 in this study.

RelA/p65 is acetylated in vitro by p300 or CBP at lysine 310, 314 and 315.

To identify the acetylation residues*, in vitro* acetylated RelA/p65 by p300 was digested with trypsin and the resulting peptides were analyzed by LC/MS/MS. 81.8% of the K310 comprising peptides contained an acetylated K310. 61.9% of K314 and 56.52% of K315 containing peptides showed acetylated K314 and acetylated K315, respectively. These data indicate that lysine 310, 314 and 315 were acetylated by p300. The identified lysine residues are located close to the C-terminus of the Rel homology domain (RHD) (Fig. 2*A*). To confirm these findings, the corresponding lysines were replaced with arginine residues by site directed mutagenesis. Substitution of lysine to arginine maintains the positive charge of the residue and may cause only minimal changes in the local environment of the protein. Wild type or mutated RelA/p65 harboring K310R, K314/315R or all three K310/314/315R (KTR) substitutions were expressed and purified from insect cells (Fig. 2*B*). Subsequently, all proteins were subjected to *in vitro* acetylation by p300 or CBP (Fig. 2*C*). Acetylation of RelA/p65 mutated at single K310 or K314/315 was only slightly reduced compared to wild type, while mutation of all three lysine residues abolished acetylation of RelA/p65 (Fig. 2*C*). When the purified protein substrates were tested only in the presence of acetyl-CoA, no acetylation was observed confirming that the acetylation was mediated by p300 or CBP and not by a co-purified acetyltransferase. These results clearly indicate that K310, K314 and K315 of RelA/p65 are the main acetylation sites of p300 and CBP *in vitro*. To confirm that the RelA/p65KTR mutant was still able to interact with p300, myc-tagged RelA/p65 wild type and RelA/p65K/R mutant proteins were overexpressed in HEK 293T cells that were treated with PMA to induce NF-κB. RelA/p65 was immunoprecipitated with an anti-myc antibody and the immunoprecipitated complexes were analyzed by western blot analysis using an anti-p300 antibody. Interaction of p300 with the RelA/p65KTR mutant was similar to that observed for the RelA/p65 wild type (Fig. 2*D*). To further confirm these findings, GST-pull down experiments were performed using GST-RelA/p65 wild type or GST-RelA/p65KTR as bait proteins in the presence of purified p300. Subsequent western blot analysis revealed that p300 was able to equally interact with both RelA/p65 wild type and the RelA/p65KTR mutant (Fig. 2*E*). These results indicate that abolished acetylation of the RelA/p65KTR mutant was due to the lack of specific sites but not due to the inability of this mutant to interact with p300.

RelA/p65KTR is transcriptionally active in vitro.

To investigate whether acetylation of RelA/p65 might influence its potential to activate gene expression, *in vitro* transcription assays on chromatinised templates were performed (Fig. 3*A*). In these experiments the RelA/p65 wild type and the RelA/p65KTR were added to template DNA (HIV-LTR-luc) after chromatin assembly (Fig. 3*B*). Low basal transcription was detected in the absence of the activator proteins. When we added recombinant RelA/p65 wild type or RelA/p65KTR to the reaction, transcription was activated to similar extent by both proteins. These results suggested that the triple mutation in RelA/p65KTR per se did not affect the overall structure of RelA/p65. p300 substantially stimulated the transcription from chromatinised templates in the presence of RelA/p65 wild type and RelA/p65KTR. Such stimulation was not evident when p300 was added alone to the reaction. The level of p300-induced transcription was not significantly different between the RelA/p65wt and the RelA/p65KTR. These results indicate that acetylation of K310, K314 and K315 by p300 did not affect RelA/p65-dependent transcription initiation from the synthetic HIV-LTR reporter.

RelA/p65 is acetylated in vivo in TNFα stimulated cells.

To investigate the role of RelA/p65 acetylation of K310, K314 and K315 *in vivo,* RelA/p65-/- NIH 3T3 mouse embryonic fibroblasts (MEF) were genetically complemented using lentiviruses encoding myc-RelA/p65 wild type, myc-RelA/p65K310R, myc-RelA/p65K314/315R or myc-RelA/p65KTR. After appropriate selection cells were kept in pools and the expression of recombinant proteins was analyzed by western blotting using an anti-RelA/p65 antibody (Fig. 4*A*). The cells transduced with control virus encoding the resistance gene (mock infected) and non-transduced wild type NIH 3T3 cells expressing endogenous RelA/p65 protein were included as controls. The expression levels of the recombinant wild type and mutated

RelA/p65 proteins were comparable to that observed for endogenous RelA/p65 in NIH 3T3 cells. Furthermore, analysis of the cell growth revealed that the proliferation rate was comparable between the tested cell pools under normal growth conditions (data not shown).

It was shown before that RelA/p65 was acetylated in a stimuli dependent manner (28). In order to investigate whether RelA/p65 is acetylated at the identified lysine residues *in vivo*, antibodies directed against p65 peptides acetylated at residues 310, 314 or 315 were generated. Nuclear extracts were prepared after treating the complemented cells with TNFα and HDAC inhibitors (TSA and NAM). RelA/p65 nuclear import upon TNFα treatment was induced in RelA/p65wt and RelA/p65 mutant complemented cells (Fig. 4*B*). Immunoprecipitation with an anti-RelA/p65 antibody and subsequent western blot analysis using the specific acetyl K310 antibody revealed that RelA/p65 was indeed acetylated at this site upon TNFα treatment (Fig. 4*C*). Antibodies raised against acetylated 314 and acetylated 315 were found to be unspecific in this analysis (data not shown).

Neither shuttling nor DNA binding of RelA/p65 is affected by the K/R substitution mutation.

The complemented cells were treated with TNFα and subcellular localization of the recombinant RelA/p65 proteins was analyzed by immunofluorescence analysis at different time points (Fig. 5A). Nuclear translocation of the recombinant RelA/p65 wild type was detected after 20 minutes of TNFα treatment and relocation to the cytoplasm after 60 minutes. A similar shuttling kinetics was observed for endogenous RelA/p65 protein in NIH 3T3 cells (data not shown). Analysis of the cells complemented either with K310R, K314/315R or KTR revealed no significant differences in the shuttling kinetics between the mutated RelA/p65 and the RelA/p65 wild type proteins (Fig. 5*A*). This suggested that acetylation of RelA/p65 at the three tested lysine residues was not essential for the regulation of the RelA/p65 nuclear-cytoplasmic redistribution. This observation is supported by our findings that IκBα was able to bind to acetylated and non-acetylated RelA/p65 with similar affinity (data not shown).

Next we investigated the influence of p300-mediated RelA/p65 acetylation on the ability of RelA/p65 to bind DNA. Nuclear extracts of the complemented cells treated with TNFα were tested in an electro mobility shift assay using an oligonucleotide containing two κB elements of the HIV-LTR promoter. The experiments revealed that mutations of K310, K314 and K315 did not significantly influence the TNFα induced DNA binding of RelA/p65 (Fig. 5*B*). Binding was substantially reduced by competition with non-labeled oligonucleotides containing a wild type κB site, but not a mutated κB site, indicating that the binding was specific (data not shown). The presence of RelA/p65 and p50 in the complex was confirmed by supershift experiments using specific anti-RelA/p65 and anti-p50 antibodies, respectively (Fig. 5*B*).

Determination of genes regulated by p300-mediated acetylation of RelA/p65.

The important question was to investigate whether the acetylation deficient mutants would affect endogenous gene expression in TNFα treated cells. To determine which, if any, RelA/p65-target genes depend on acetylation of K310, K314 and K315 we performed microarray analysis (Agilent) with oligonucleotides representing annotated genomic mouse sequences. RelA/p65-/- cells complemented either with wild type or KTR mutant were treated for 45 minutes with TNFα and total RNA was isolated in three replicates from these cells. RNA was then amplified and co-hybridized to the Agilent mouse whole genome array. Statistical analysis of the expression profiles of cells complemented with wild type or KTR RelA/p65 revealed differentially expressed genes. Among them 28 genes were up regulated more than 1.6 (log) fold with a p value ≤0.01 (Table 1) and 32 genes down regulated more than 0.625 with a p value ≤0.01 in cells expressing RelA/p65KTR when compared to RelA/p65 wild type cells (Table 2). Since most of the regulated genes were previously not identified as NF-κB target genes, the upstream genomic regions (9kb) were searched for the presence of a NF-κB consensus DNA binding site (5'-GGGRNNYYCC-3') using the GeneSpring program (Fig. 6*A*). We found that 68% of the up regulated genes and 72% of the down regulated genes contained a consensus NF-κB binding site. Statistical test revealed a significant overlap between the regulation of these genes and the presence of the κB site (Fig. 6*A*).

To confirm the results derived from the

microarray studies, we further analyzed the expression of several genes with quantitative RT-PCR. To perform quantitative real-time RT-PCR total RNA isolated from RelA/p65-/- cells complemented either with wild type or KTR mutant was reverse transcribed with random hexamer primers to obtain cDNA. In these experiments the cells were stimulated with TNFα for 45 minutes. As expected from the microarray analysis Mip-2 expression was induced to the same extent in RelA/p65 wild type and RelA/p65 KTR complemented cells (Fig. 6*B,* left). However, Ccl-7 expression was increased (2 fold, p value=0.0695) in cells expressing KTR mutant confirming that lack of acetylation at these sites has a positive effect on its regulation (Fig. 6*B,* middle). Finally, Fhl-1 expression was highly dependent on the acetylation of RelA/p65 (p=0.0123), since expression of the Fhl-1 gene could not be detected in cells expressing the KTR mutant (Fig. 6*B*, right). Therefore, the results obtained in the microarray analysis could be confirmed by the more sensitive real time RT-PCR method for three selected genes. Together, this analysis provides strong evidence that acetylation of RelA/p65 at lysine 310, 314 and 315 is required for the transcriptional regulation of a specific subset of genes.

DISCUSSION

Increasing experimental evidence has indicated that NF-κB-dependent gene expression is regulated by different post-translational modifications including acetylation (37). The acetylation-dependent NF-κB regulation was shown to occur via many different mechanisms (38). For example acetylation of histones is known to regulate NF-κB-dependent gene accessibility for the transcriptional machinery (37). The direct acetylation of NF-κB was reported to regulate the transcriptional potential of NF-κB, the duration of the NF-κB response, its DNA binding activity as well as protein-protein interactions with several transcription cofactors (31,39,40).

In this study we have addressed the role of p300-mediated acetylation of RelA/p65 in the regulation of gene expression *in vivo*. We identified three lysines (K310, K314 and K315) in RelA/p65 as targets for the acetylation by p300 *in vitro* (Fig. 2*A*). Interestingly, K310 was previously shown to be acetylated by p300 (31). However, we additionally found two new lysine residues acetylated in RelA/p65 that were not reported before. It is important to mention that among the previously reported acetylation sites (29,31) we could only confirm the acetylation of K310 in our experimental system. This could be due to the different experimental procedures used in the studies. Therefore, we cannot exclude that additional p300 specific acetylation sites exist in RelA/p65. The relevance of the *in vitro* acetylation sites identified in our study was confirmed by the ability of RelA/p65 to be acetylated *in vivo* upon TNFα stimulation (Fig. 4*C*).

We also demonstrated by an *in vitro* transcription assay on chromatinised templates that both RelA/p65 wild type and RelA/p65KTR (K310R, K314R, K315R) are transcriptionally active and can be stimulated by p300 (Fig. 3B). Since the p300-mediated increase in transcription initiation was dependent on the presence of RelA/p65 proteins, this may suggest that RelA/p65 facilitates the recruitment of p300 to the template. RelA/p65 is known to interact with p300 through its Rel homology and transactivation domains (25). Because a similar increase in transcription was seen for both the wild type and the acetylation deficient RelA/p65 mutant, we conclude that acetylation of RelA/p65 was not important for the stimulation. We could not exclude that acetylation of other proteins by p300 was responsible for the transcriptional up regulation in this system. p300 was previously reported to acetylate nucleosomal histones on chromatin templates (41) and is known to acetylate other non-histone proteins (42) .

To investigate the role of RelA/p65 acetylation in endogenous gene expression *in vivo* we genetically complemented RelA/p65-/- fibroblasts with a control vector or cDNAs encoding for RelA/p65 wild type, K310R, K314/315R and KTR mutant (Fig. 4*A*). Analysis of the complemented cells revealed that the mutation of the acetylation sites did not affect the kinetics of the cytoplasmic-nuclear redistribution of RelA/p65 upon TNFα stimulation (Fig. 5*A*). This implied that the upstream signalling events are not regulated by the acetylation of RelA/p65 at the identified sites.

To elucidate the influence of RelA/p65 acetylation on gene expression *in vivo* we performed genome-wide microarrays using total RNA isolated from the complemented cells. We

identified subsets of genes which were specifically up or down regulated depending on the ability of RelA/p65 to be acetylated at the described sites (Table 1, 2). The majority of these genes were previously not described as NF-κB target genes. However, the presence of a NF-κB consensus site in their upstream regions indicated that they could be regulated by NF-κB. The results of the microarray analysis revealed that the acetylation of RelA/p65 does not always correlate with activation of gene expression as has been suggested before (31). Rather we provide evidence that the p300-mediated acetylation of RelA/p65 contributes to both gene specific activation and repression of transcription. It is unknown how RelA/p65 acetylation signals to activate certain genes while simultaneously suppressing the expression of others. One hypothesis would be that the regulation of certain genes by p300-mediated acetylation of RelA/p65 promotes the recruitment of additional factors or stabilizes the formation of specific preinitiation complexes at promoter sites. Like this acetylated RelA/p65 could serve as a factor that regulates the recruitment of these proteins. It has been previously reported that many proteins can specifically recognize and bind acetylated lysine residues in histones and transcription factors through their bromo domains (43-45). Among them are well characterized histone acetyltransferases (e.g., p300, P/CAF and GCN5) (46) and subunits of chromatin remodelling complexes (e.g., ATPases of SWI2/SNF2 and proteins of the WAL/BAZ family of ISWI-associated proteins) (47). The nature of the recruited co-activator/co-repressor would determine the response of the specific genes. Another hypothesis would be that the presence of other cis-regulatory elements in a targeted gene and regulatory proteins recruited to these elements might be critical for the modulation of the acetylation dependent NF-κB response. Acetylation is known to be a reversible protein modification. In this regard HDAC-1, HDAC-2 and HDAC-3 were reported to repress NF-κB-dependent transcription upon treatment with inflammatory stimuli (31,37,38). These histone deacetylases were also shown to directly interact with several proteins involved in the NF-κB signalling pathway, including NF-κB itself (28,37,48). Deacetylation of RelA/p65 by HDAC-3 may provide a counterpart mechanism for p300 function. The exact molecular mechanism by which acetylation/deacetylation of RelA/p65 regulates the transcription activity of RelA/p65 in the context of chromatin remains to be investigated.

Together, acetylation of RelA/p65 presents an attractive regulatory mechanism for the control of NF-κB dependent gene expression. The combination of acetylation with other post-translational modifications will even more broaden the potential of this regulation. Combinations of different modifications have already been proposed to serve as a 'code' for the interacting domains of different proteins (49,50). Furthermore, acetylation can be regulated by other post-translational modifications. For example phosphorylation of RelA/p65 at serine 276 and 536 was shown to enhance the acetylation of lysine 310 (51). It cannot be excluded that other post-translational modifications of RelA/p65 can also regulate the acetylation of this transcription factor. Our findings could help to explain the diversity of NF-κB-dependent gene expression upon different stimuli. We hypothesize that unique combinations of post-translational markers and the presence of cell type specific cofactors determine the specific NF-κB-mediated response.

In conclusion, our results support the hypothesis that p300-mediated acetylation of RelA/p65 is important to modulate the expression of defined genes, thereby contributing to the specificity of the NF-κB response.

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FIGURE LEGENDS

Fig. 1. RelA/p65 is acetylated by p300 and CBP in *vitro*. *A*, Recombinant histone acetyltransferases (HATs) expressed and purified from insect cells were analyzed by SDS-PAGE and Coomassie staining. Molecular weight markers are shown on the left. *B*, *In vitro* acetylation assay using full-length RelA/p65 and the indicated HATs. RelA/p65 was incubated in the presence of radioactively labeled [^{14}C]-acetyl-CoA with 500ng of p300 or CBP or the equimolar amount of GCN5L, P/CAF or Tip60. Proteins were resolved on SDS-PAGE, stained with SyproRuby (left) and exposed to X-ray films (right). RelA/p65 acetylation signals are indicated with an arrow. HATs or HAT autoacetylation signals are indicated with an asterisk (*). *C*, *In vitro* acetylation of histones. *In vitro* acetylation assay was performed as described in *B*.

Fig. 2. RelA/p65 is acetylated by p300 at lysine 310, 314 and 315 *in vitro*. *A,* Protein domain diagram of RelA/p65 depicts the location of the acetylated lysine residues in the 'linker' region of RelA/p65 (NLS, nuclear localization signal; RHD, Rel homology domain; TAD, transactivation domain). *B,* Coomassie staining of recombinant arginine to lysine substitution mutants of RelA/p65 expressed and purified from insect cells: wt, RelA/p65 wild type; K310R, RelA/p65K310R; K314/315R, RelA/p65K314/315R and KTR, RelA/p65K310/314/315R. *C, In vitro* acetylation of RelA/p65K/R mutants by p300 or CBP. Proteins were analyzed as in Fig. 1*B*. The autoacetylation signals of p300 or CBP are indicated with an asterisk (*). *D,* Analysis of interaction of recombinant RelA/p65 proteins with p300 in PMA treated HEK293T cells. Wild type myc-RelA/p65 and myc-RelA/p65 K/R mutants were immunoprecipitated from whole cell extract of PMA treated HEK 293T cells using anti-myc antibody. Immunocomplexes were resolved on SDS-PAGE and subsequently analyzed by western blot with anti-p300 and anti-myc antibodies. *E,* GST-pull down experiments using GST, GST-RelA/p65wt and GST-RelA/p65KTR as bait proteins were performed in the presence of purified his-p300. Co-precipitated proteins were analyzed by western blot using anti-his antibody. Coomassie staining of the bait proteins for equal loading is shown on the right.

Fig. 3. RelA/p65KTR is transcriptionally active *in vitro*. *A,* Schematic representation for chromatin assembly and transcription. Chromatin was assembled with template DNA, S190 extract and core histones (CH). Transcription pre-initiation complex (PIC) formation was performed with HeLa

nuclear extract (NE) and transcription was initiated by adding rNTPs. RelA/p65 and p300 were added after chromatin assembly but before PIC formation. *B,* Transcriptional activation by RelA/p65wt or RelA/p65KTR on chromatin templates using *in vitro* transcription assays with the HIV-LTR DNA template. p300 was added at the indicated time point. The transcripts were detected with primer extension. The average mean with standard deviations of three independent experiments is shown below.

Fig. 4. RelA/RelA/p65 is acetylated at lysine 310 *in vivo* in response to TNFα. *A,* Western blot analysis with anti-RelA/p65 antibody of whole cell extracts from RelA/p65-/- MEFs complemented with RelA/p65 wild type (wt) and the different substitution mutants of RelA/p65 (K310R, K314/315R and KTR). Mock transduced and NIH 3T3 cells were used as controls. The membrane was reprobed with anti-tubulin antibody as loading control. *B,* Nuclear extracts of the complemented cell lines untreated or treated with TNFα (30ng/ml) for 30 minutes were subjected to western blot analysis using anti-RelA/p65 antibody. The anti-PARP western blot was performed to check the extract fractionation. *C,* Nuclear extracts of the complemented cell lines treated with TNFα and HDACi (TSA/NAM) were subjected to immunoprecipitation analysis using anti-RelA/p65 antibodies. Western blot analysis with the anti-RelA/p65ac310 antibody was performed. The membranes were reprobed with anti-RelA/p65 antibody.

Fig.5. Mutation of the acetylation sites in RelA/p65 does not affect its nuclear translocation and DNA binding. *A,* The complemented cells were treated with TNFα and fixed with paraformaldehyde followed by immunostaining using anti-RelA/p65 antibodies. Subcellular localization of RelA/p65 protein was analyzed by immunofluorescence microscopy. *B,* Electro mobility shift assays were performed with nuclear extracts from the untreated or TNFα treated cells using ^{32}P-labeled oligonucleotide DNA containing 2κB sites. The bound complexes were characterised by anti-RelA/p65 or anti-p50 supershift assays. Specific NF-κB complexes are indicated with an arrow, unspecific bands are marked with an asterisk (*).

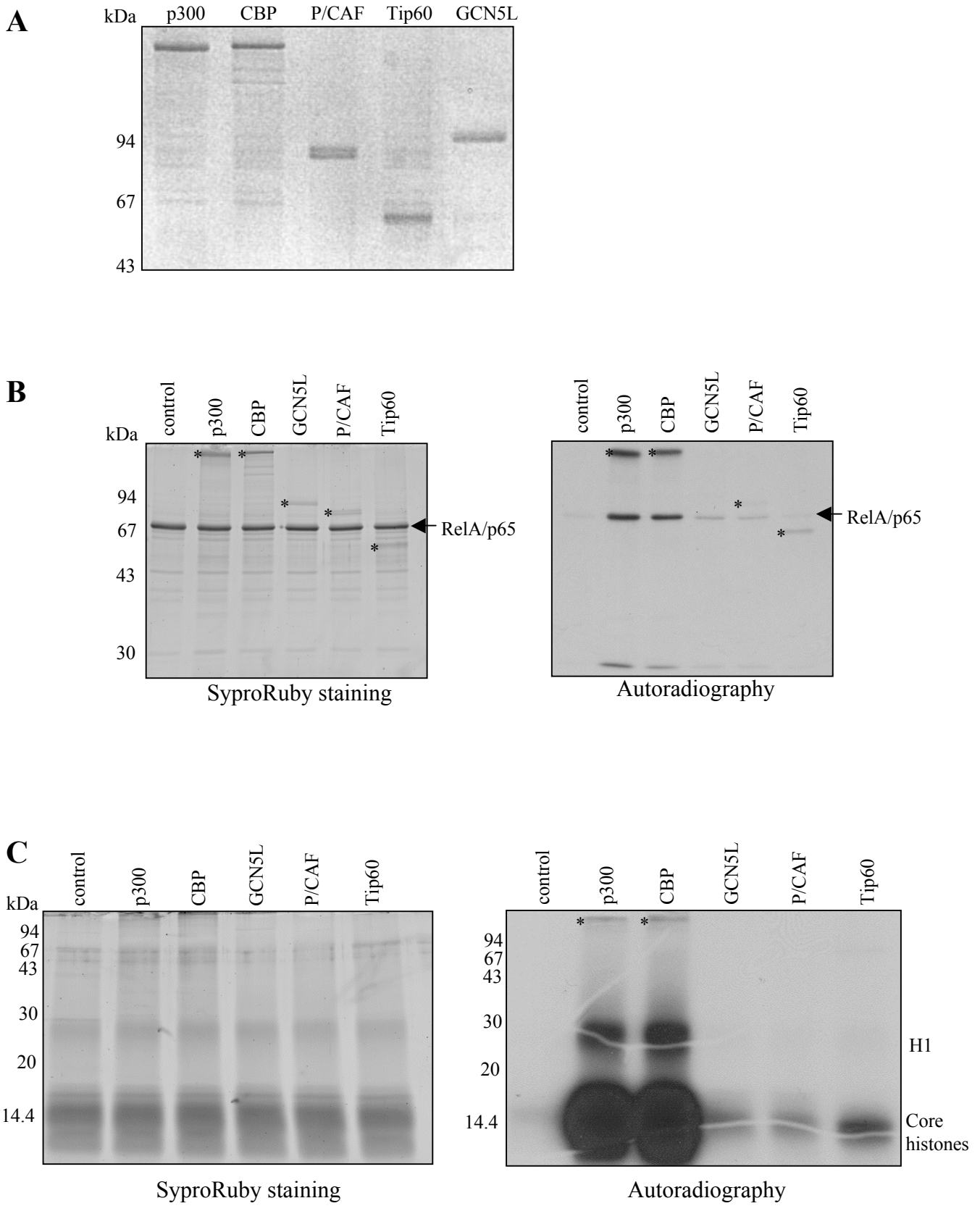
Fig.6. Acetylation of RelA/p65 at lysine 310, 314 and 315 influences gene specific transcription.
A, Venn diagram showing the overlap between the regulated genes found in the microarray analysis and the presence of a consensus NF-κB site in their 9 kb upstream region. The overlap is non-random (p (up-regulated)=0.0228 and p (down-regulated)=0.00283). *B,* Quantitative real time RT-PCR using TaqMan probes for three selected genes (Mip-2, Ccl-7 and Fhl-1). The 18SrRNA was used to correct for differences in cDNA inputs. The results of three independent experiments with standard deviations are shown.

Table 1. Upregulated genes, KTR(45')/ wild type (45'), fold change>1.6, p≤0.01

Gene name	Description	Genbank ID	Fold change	p value	NF-κB site (+1 - -9000)
Col4a6	Collagen type IV	BC057648	11.74	0.00177	Y
Peg12	Paternally expressed 12	NM_013788	6.696	0.000929	N
Dlk1 (SCP1)	Delta-like homolog 1	D16847	5.408	0.000811	N
Riken cDNA	A830059I20	NM_021427	4.859	0.00104	Y
Dlk1	Delta-like homolog 1	NM_010052	4.703	0.000313	N
Frat1	Frequently rearranged in advanced T-cell lymphomas	NM_008043	4.577	0.00166	N
Ptpns1	Tyrosine-protein phosphatase non-receptor substrate type 1	D87968	2.871	0.00613	Y
Kiaa1914	Riken cDNA AK087449	NM_146102	2.855	0.0088	Y
Plf	Proliferin, short variant	X75557	2.797	0.00553	N
Mrpplf4	Mitogen related protein, proliferin 4	NM_181852	2.598	0.00387	Y
Plf2	Proliferin 2	NM_011118	2.571	0.00507	N
Vegfc	Vascular endothelial growth factor C	NM_009506	2.286	0.002	N
Serpinb6b	Serine proteinase inhibitor, clade B, member 6B	NM_011454	2.277	0.00769	Y
Gcnt1	Glucosaminyl (N-acetyl)transferase 1,core 2	NM_173442	2.207	0.000969	Y
Uchl1	Ubiquitin carboxyterminal hydrolase L1	NM_011670	2.167	0.000528	N
Riken cDNA	Similar to ARGBP2A (human)	A530071H08	2.116	0.00439	N
Lama5	Laminin alpha 5 chain	U37501	2.111	0.00413	Y
Msrb2	Methionine sulfoxide reductase B	NM_029619	2.097	0.00328	Y
Cdyl2	Chromodomain protein, Y chromosome–like 2	NM_029441	2.003	0.00526	Y
Prkg2	Protein kinase, cGMP-dependent type II	NM_008926	1.994	0.00293	N
Riken cDNA	Clone 3110013H01	AK01040460	1.939	0.0072	N
Uchl1	Ubiquitin carboxyterminal hydrolase L1	NM_011670	1.862	0.00566	N
Lrrc5	Leucine-rich repeat containing protein 5	NM_178701	1.859	0.00804	Y
Riken cDNA	Ionized calcium binding adapter molecule 2 (Iba2)	BC024599	1.796	0.00666	Y
Mgst1	Microsomal glutathione S-transferase 1	AK002800	1.789	0.00754	Y
Dusp9	Dual specificity phosphatase 9	NM_029352	1.779	0.0092	Y
Gcnt1	Glucosaminyl(N-acetyl)transferase 1,core 2	NM_010265	1.76	0.0028	Y
Ccl-7	Chemokine (C-C motif) ligand 7	NM_013654	1.717	0.00792	Y
Bcl6b	B-cell CLL/lymphoma 6, member B	NM_007528	1.66	0.0032	Y
Ssbp4	Single stranded DNA binding protein 3-homolog	NM_13372	1.643	0.00493	Y
Baz1b	Bromodomain adjacent to zinc finger domain, 1B	NM_011714	1.634	0.00456	Y
Npepl1	Aminopeptidase-like 1	NM_213733	1.629	0.00661	Y
Mgst1	Microsomal glutathione S-transferase 1	NM_019946	1.614	0.00802	Y
Gcnt1	Glucosaminyl (N-acetyl) transferase 1, core 2	NM_010265	1.613	0.00435	Y

Table 2. Downregulated genes: KTR (45')/ wild type (45'), fold change <0.625, p≤0.01

Gene name	Description	Genbank ID	Fold change	p value	NF-κB (+1- -9000)
Cnn1	Calponin 1	NM_009922	0.0978	0.00066	Y
Myl9	Myosin regulatory light chain 2, smooth muscle isoform	AK007972	0.112	0.00195	N
Fhl1	Four and a half LIM domains 1	NM_010211	0.23	0.00085	Y
Acta2	Actin, alpha 2, smooth muscle, aorta	A530078I09	0.284	0.00688	Y
Thy1	Thymus cell antigen 1, theta	NM_009382	0.285	0.00291	Y
Tagln	Transgelin	NM_011526	0.296	0.00666	Y
Rtp4	Receptor transporting protein 4	NM_023386	0.305	0.000914	Y
Tagln	Transgelin	NM_011526	0.34	0.0087	Y
Itgbl1	Ten integrin EGF-like repeat domains protein precursor homolog	NM_145467	0.35	0.0021	N
Bst2	DAMP-1 protein, Bone marrow stromal cell antigen 2	NM_198095	0.382	0.00964	Y
Gbp3	Guanylate nucleotide binding protein 3	NM_018734	0.404	0.00364	Y
LOC56628	MHC (A.CA/J(H-2K-f) class I antigen	NM_019909	0.454	0.00207	Y
Kcnab1	Potassium voltage-gated channel, shaker-related subfamily, beta member 1	NM_010597	0.474	0.00664	Y
Riken cDNA	Unknown EST	1700006C13	0.485	0.00224	N
H2-K1	MHC class I heavy chain precursor (H-2K(b))	U47328	0.494	0.00338	Y
Zfp286	Zink finger protein	NM_138949	0.498	0.00599	Y
Agrn	Agrin	NM_021604	0.52	0.00902	N
Hmmr	Hyaluronan mediated motility receptor	NM_013552	0.521	0.00559	N
Ak5	Adenylate kinase 5	AK053807	0.541	0.00501	N
Akap12	A kinase (PRKA) anchor protein (gravin) 12	NM_031185	0.552	0.00565	Y
Ccng1	Cyclin G1	NM_009831	0.558	1.20E-05	Y
Gpr64	G protein-coupled receptor 64	NM_178712	0.559	0.00589	Y
Hipk2	Homeodomain interacting protein kinase 2	AK003718	0.566	0.00268	N
Pmaip1	Phorbol-12-myristate-13-acetate-induced protein 1	NM_021451	0.567	0.000887	Y
Dgat2	Diacylglycerol O-acyltransferase 2	NM_026384	0.57	0.00066	Y
Cyba	Cytochrome b-245, alpha polypeptide	AK021200	0.573	0.00142	Y
Sash1	SAM and Sh3 domain containing 1	NM_175155	0.576	0.00539	N
Itpr1	Inositol 1,4,5-triphosphate receptor1	NM_010585	0.595	0.00803	Y
Gng12	Guanine nucleotide binding protein (G protein), gamma 12	NM_025278	0.601	0.00184	Y
Nudcd2	NudC domain containing 2	NM_026023	0.603	0.00103	N
Gbp2	Guanylate nucleotide binding protein 2	NM_010260	0.603	0.0086	Y
Prkcm	Protein kinase C, mu	NM_008858	0.618	0.00447	Y
Ghr	High molecular weight growth hormone receptor/binding protein	M33324	0.624	0.00245	Y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kDa
p300
CBP
P/CAF
Tip60
GCN5L
94
67
43
B
kDa
control
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CBP
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P/CAF
Tip60
94
67
43
30
RelA/p65
SyproRuby staining
Autoradiography
C
kDa
control
p300
CBP
GCN5L
P/CAF
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94
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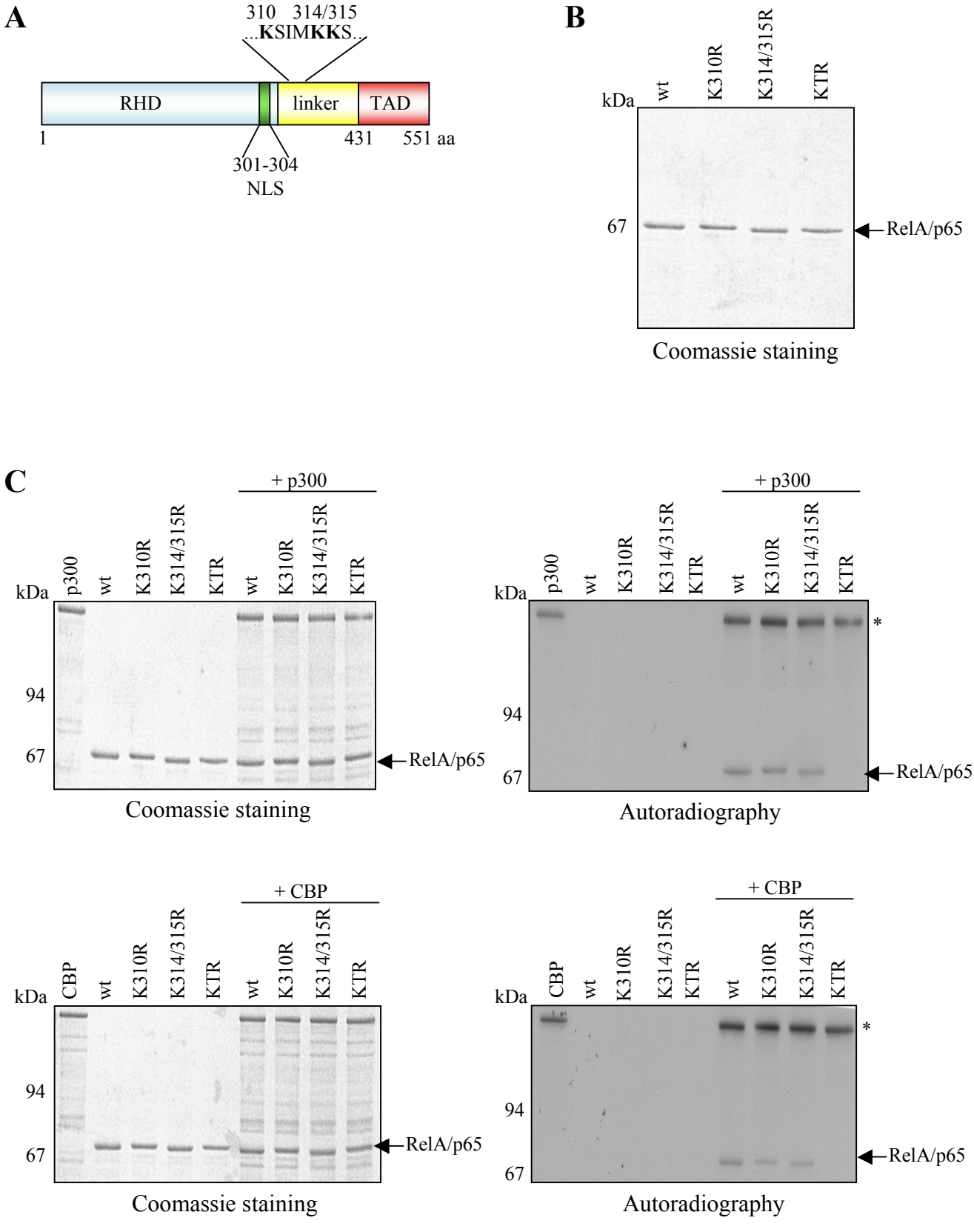
43
30
20
14.4
H1
Core
histones
SyproRuby staining
Autoradiography

Buerki *et al.*, Figure 2.



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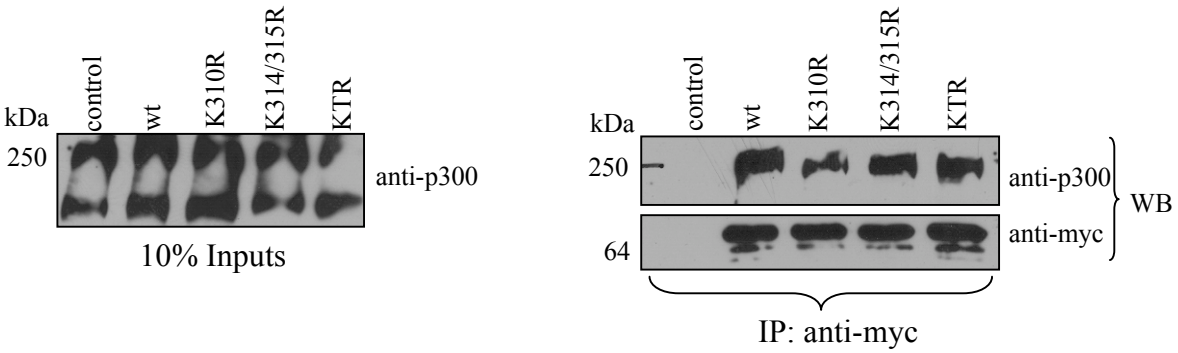
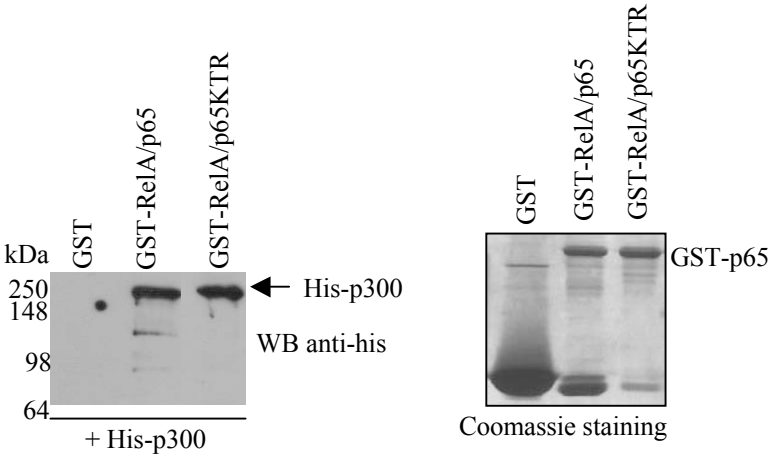

kDa
control
wt
K310R
K314/315R
KTR
250
anti-p300
10% Inputs
kDa
control
wt
K310R
K314/315R
KTR
250
anti-p300
WB
64
anti-myc
IP: anti-myc

E


kDa
GST
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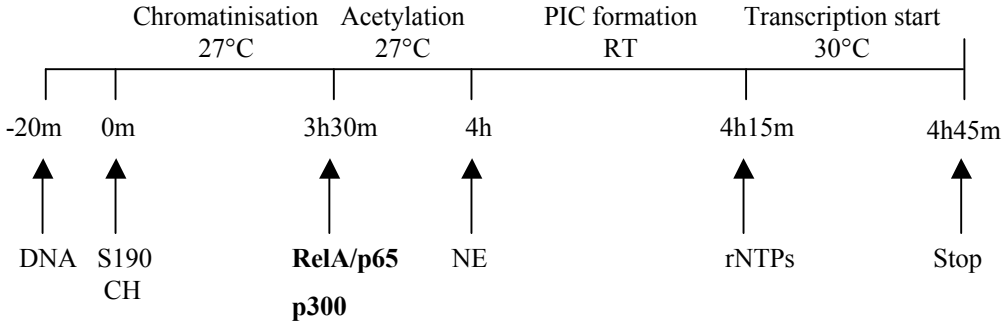
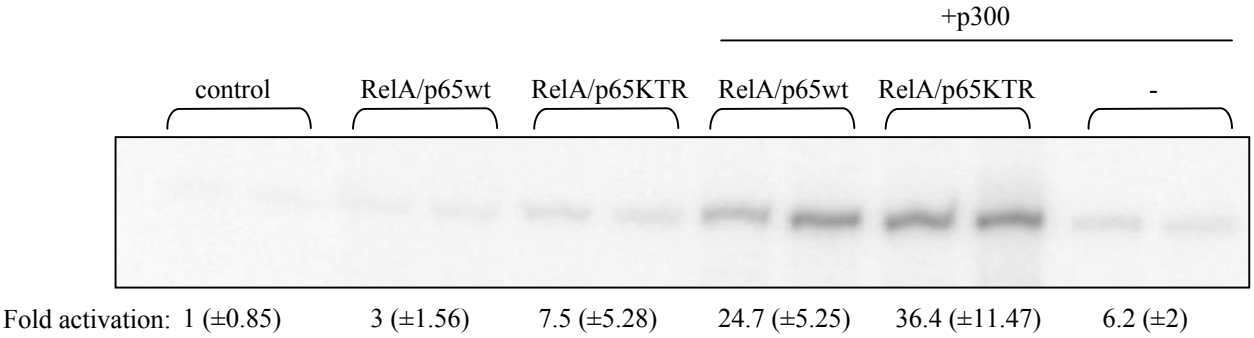
GST-RelA/p65KTR
250
148
98
64
His-p300
WB anti-his
+ His-p300
GST
GST-RelA/p65
GST-RelA/p65KTR
GST-p65
Coomassie staining

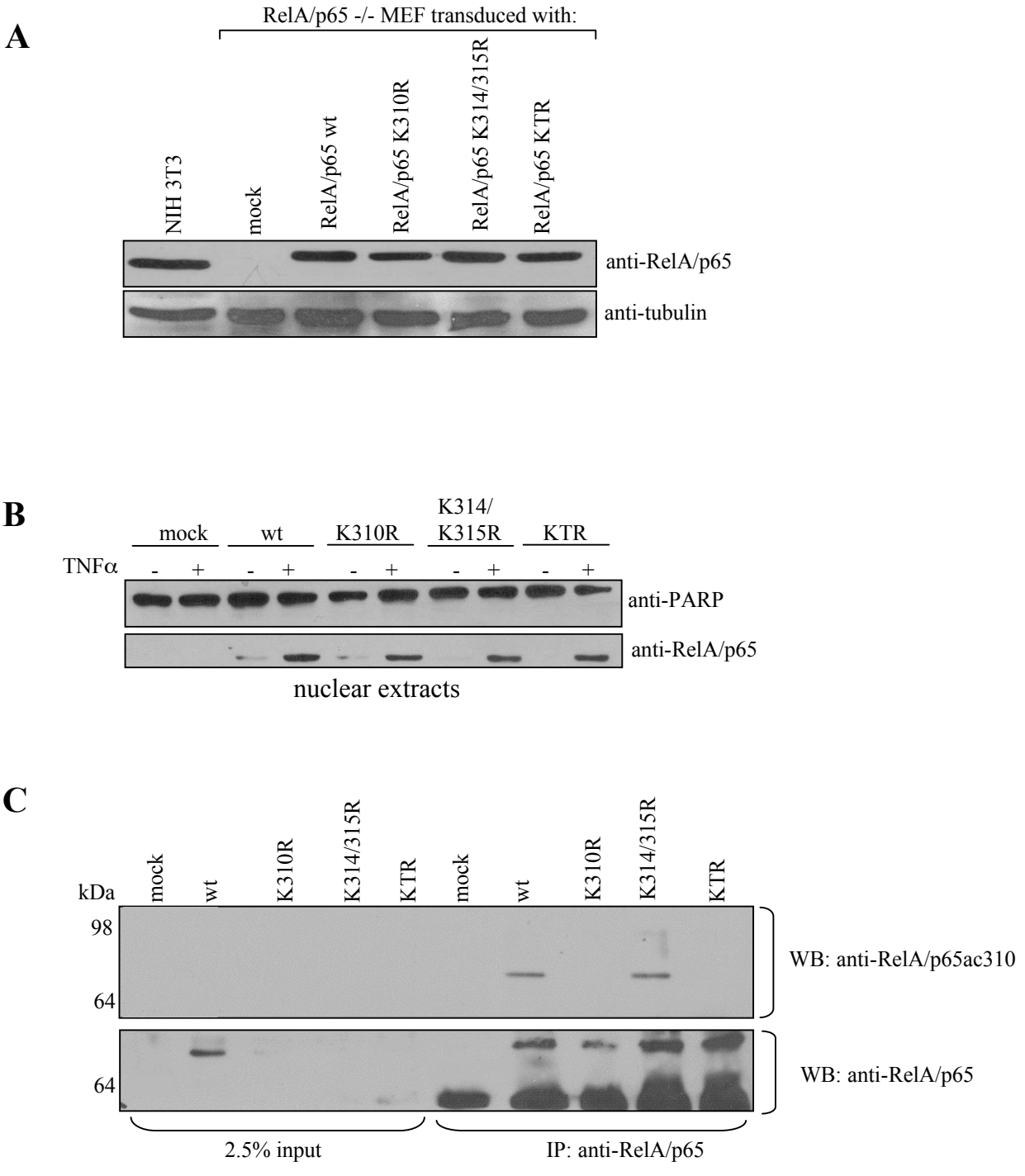
Buerki *et al*., Figure 3

A

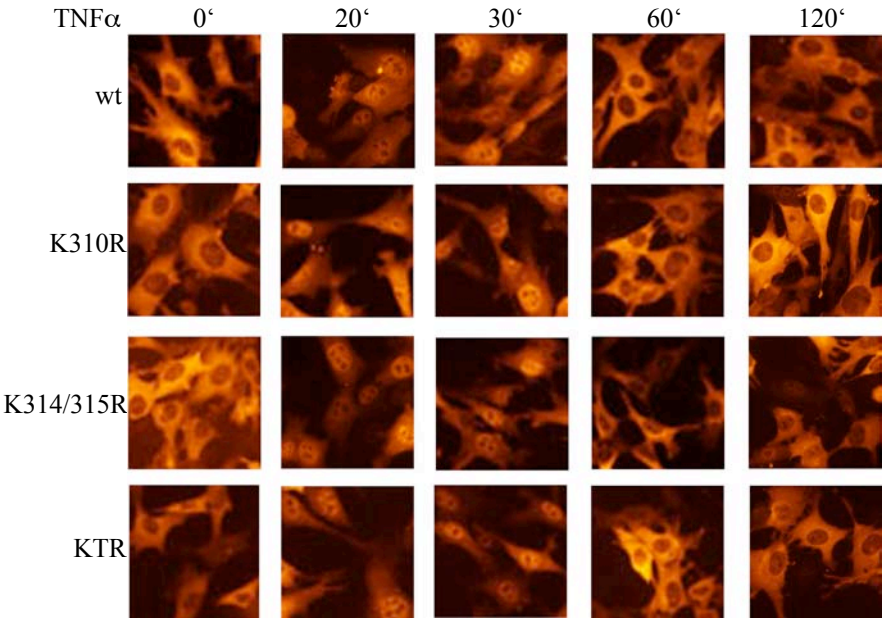


B

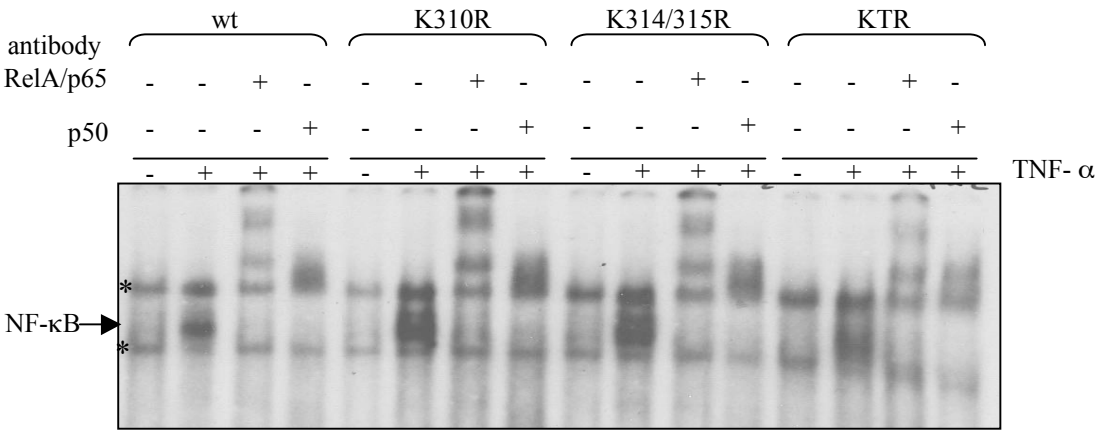



A
RelA/p65 -/- MEF transduced with:
NIH 3T3
mock
RelA/p65 wt
RelA/p65 K310R
RelA/p65 K314/315R
RelA/p65 KTR
anti-RelA/p65
anti-tubulin
B
mock
wt
K310R
K314/
K315R
KTR
TNFα
- + - + - + - + - +
anti-PARP
anti-RelA/p65
nuclear extracts
C
kDa
mock
wt
K310R
K314/315R
KTR
mock
wt
K310R
K314/315R
KTR
98
64
64
WB: anti-RelA/p65ac310
WB: anti-RelA/p65
2.5% input
IP: anti-RelA/p65

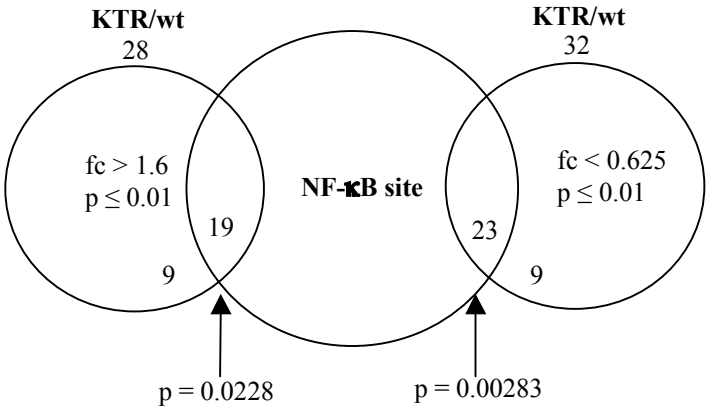
A


TNFα
0‘
20‘
30‘
60‘
120‘
wt
K310R
K314/315R
KTR

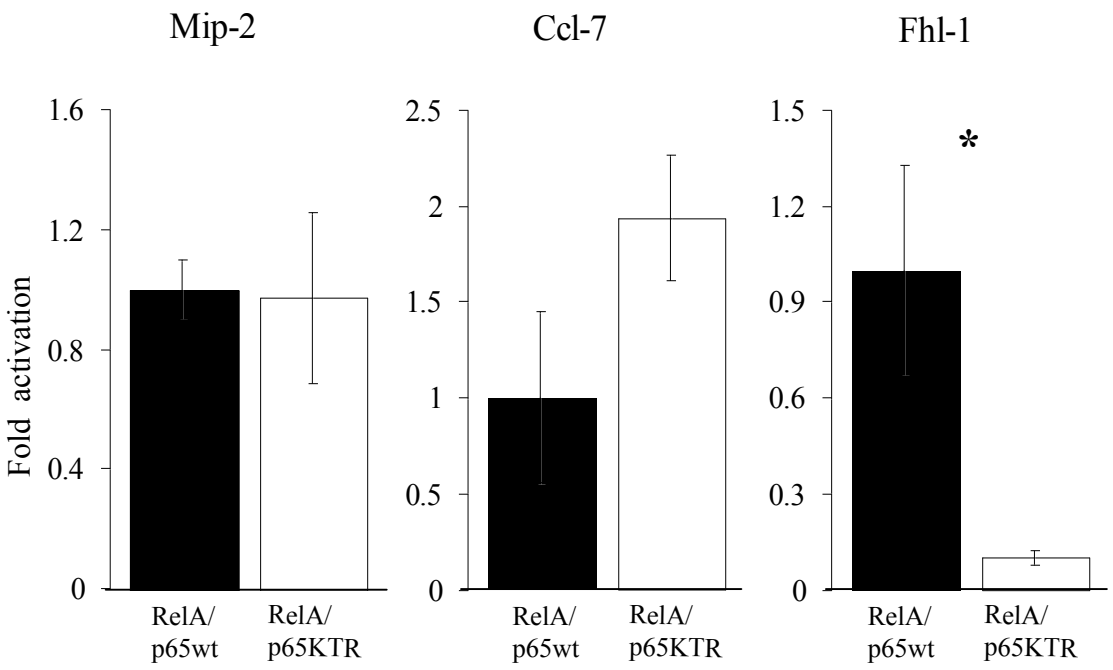
B


wt
K310R
K314/315R
KTR
antibody
RelA/p65
p50
TNF- α
NF-κB

A


KTR/wt
28
KTR/wt
32
fc > 1.6
p ≤ 0.01
NF-κB site
fc < 0.625
p ≤ 0.01
19
23
9
9
p = 0.0228
p = 0.00283

B


Mip-2
Ccl-7
Fhl-1
Fold activation
1.6
1.2
0.8
0.4
0
2.5
2
1.5
1
0.5
0
1.5
1.2
0.9
0.6
0.3
0
*
RelA/
p65wt
RelA/
p65KTR
RelA/
p65wt
RelA/
p65KTR
RelA/
p65wt
RelA/
p65KTR

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Acetylation of Poly(ADP-ribose) Polymerase-1 by p300/CREB-binding Protein Regulates Coactivation of NF-κB-dependent Transcription*[S]

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Poly(ADP-ribose) polymerase-1 (PARP-1) and nuclear factor κB (NF-κB) have both been demonstrated to play a pathophysiological role in a number of inflammatory disorders. We recently presented evidence that PARP-1 can act as a promoter-specific coactivator of NF-κB *in vivo* independent of its enzymatic activity. PARP-1 directly interacts with p300 and both subunits of NF-κB (p65 and p50) and synergistically coactivates NF-κB-dependent transcription. Here we show that PARP-1 is acetylated *in vivo* at specific lysine residues by p300/CREB-binding protein upon stimulation. Furthermore, acetylation of PARP-1 at these residues is required for the interaction of PARP-1 with p50 and synergistic coactivation of NF-κB by p300 and the Mediator complex in response to inflammatory stimuli. PARP-1 physically interacts with the Mediator. Interestingly, PARP-1 interacts *in vivo* with histone deacetylases (HDACs) 1–3 but not with HDACs 4–6 and might be deacetylated *in vivo* by HDACs 1–3. Thus, acetylation of PARP-1 by p300/CREB-binding protein plays an important regulatory role in NF-κB-dependent gene activation by enhancing its functional interaction with p300 and the Mediator complex.

Nuclear factor κB (NF-κB) is a widely expressed transcription factor of particular importance to the regulation of cells of the immune system (1). NF-κB encompasses a family of inducible transcription factors including RelA/p65, RelB, c-Rel, p50, and p52 (1). These proteins share a conserved 300-amino acid region within their amino termini, designated Rel-homology domain (RHD). This domain is responsible for dimerization, nuclear translocation, DNA binding, and interaction with heterologous transcription factors (1). NF-κB is composed of homo- or heterodimers with a range of DNA binding and activation potentials. The most abundant and best-studied form of NF-κB in cells is a heterodimer consisting of the two subunits, p50 (NF-κB1) and p65 (RelA). NF-κB plays a key role in the regulation of many genes involved in mammalian immune and inflammatory responses, apoptosis, cell proliferation, and differentiation (1, 2). NF-κB has additionally been associated with neurodegenerative processes and cancer (3, 4). In unstimulated cells, NF-κB is sequestered in the cytoplasm as an inactive transcription factor complex by its physical association with one of several inhibitors of NF-κB (IκBs)[2] (5). Treatment of cells with extracellular stimuli including cytokines, bacterial lipopolysaccharides (LPS), phorbol esters, or potent oxidants leads to rapid phosphorylation of IκBα, which results in ubiquitination of IκBα and subsequent degradation by the 26 S proteasome (4, 5). Dissociation of NF-κB unmasks the nuclear localization sequences of p65 and p50 subunits, which leads to nuclear translocation and binding of NF-κB to specific κB consensus sequences in the chromatin and activation of specific subsets of genes (3).

NF-κB-dependent gene expression requires growing families of transcriptional coactivators (6, 7). The two key coactivators of NF-κB, histone acetyltransferases p300 and its homolog, the cAMP-response element-binding protein (CREB)-binding protein (CBP), directly associate with the NF-κB subunits p50 and p65 (8–10). These coactivators are thought to promote the rapid formation of the pre-initiation and re-initiation complexes by bridging the sequence-specific activators (like NF-κB) to the basal transcription machinery, thereby facilitating multiple rounds of transcription (11). Additionally, the histone acetyltransferases p300 and CBP can modify the amino-terminal tails of nucleosomal histones, thereby altering the local chromatin structure (12–14). Although the recruitment of p300 or CBP to NF-κB-dependent enhanceosomes is required for synergistic activation, tethering p300/CBP alone to the promoter through NF-κB is not sufficient for full activity of NF-κB in the context of chromatin. Several reports indicated that the combined actions and interactions of distinct transcriptional coactivator complexes and cofactors seem to be attributable to the strong transcriptional activity of NF-κB, depending on the stimuli and the cell type (6, 8, 15, 16).

Poly(ADP-ribose) poly-merase-1 (PARP-1) is a nuclear chromatin-associated protein and belongs to a large family of enzymes that can synthesize polymers of ADP-ribose units by using β-nicotinamide adenine dinucleotide (NAD^+) as substrate (17). PARP-1 and NF-κB have both been suggested to play a pathophysiological role in a number of inflammatory disorders (17). Several studies showed that PARP-1(−/−) mice were protected against myocardial infarction, streptozotocin-induced diabetes, LPS-induced septic shock, zymosan-induced vascular

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental material.

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[2] The abbreviations used are: IκB, inhibitor of NF-κB; CBP, cAMP-response element-binding protein (CREB)-binding protein; PCAF, p300/CBP-associated factor; AcK, lysin-acetylation domain; LPS, lipopolysaccharide; PARP-1, poly(ADP-ribose) polymerase-1; TNF, tumor necrosis factor; IFN, interferon; iNOS, inducible nitric oxide synthase; HDAC, histone deacetylase; GST, glutathione *S*-transferase; CMV, cytomegalovirus; CDK, cyclin-dependent kinase; NAM, nicotinamide; siRNA, small interfering RNA; USA, upstream stimulatory activity; MIP-2, macrophage inflammatory protein-2; TSA, trichostatin A; PCAF, p300/CBP-associated factor; HMG, high mobility group; KC, keratinocyte-derived cytokine.

failure, a non-septic model of multiple organ dysfunction as well as collagen-induced arthritis. This indicates that PARP-1 has a vital role in inflammatory disorders (17). PARP-1 has been suggested to act as a promoter-specific coactivator of NF-κB in these inflammatory disorders (18). Indeed, up-regulation of subsets of inflammatory mediators such as TNFα, IFNγ, and iNOS was shown to be impaired in PARP-1(−/−) mice upon treatment with LPS, zymosan, or streptozotocin (17, 19). We recently presented evidence that PARP-1 can act as a coactivator of NF-κB *in vivo* (18). Neither the nuclear translocation nor the DNA binding ability of NF-κB was affected in PARP-1(−/−) cells (18). However, PARP-1 directly interacted with both subunits of NF-κB (p65 and p50) *in vitro* and *in vivo* (18, 20). Remarkably, the enzymatic activity of PARP-1 was not required for full activation of NF-κB in response to various stimuli *in vivo* (20). In addition, PARP-1 directly interacted with p300 and synergistically coactivated NF-κB-dependent transcription (8). Tulin and Spradling (21) recently found that *Drosophila* mutants lacking normal PARP-1 levels display immune defects similar to mice lacking the NF-κB subunit p50. Their results imply that the role of PARP-1 in NF-κB-dependent gene expression during immune responses has been conserved during evolution.

Because NF-κB-dependent gene expression requires post-translational modifications (22), we decided to test whether the coactivator activity of PARP-1 might also be regulated by post-translational modifications. We show here that PARP-1-dependent gene expression not only requires the enzymatic activity of p300/CBP but also that PARP-1 itself is acetylated *in vivo* in response to inflammatory stimuli. Acetylation sites were mapped *in vitro* and *in vivo* to Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524. Furthermore, acetylation of PARP-1 at these lysines is required for the interaction with p50 and the transcriptional activation of NF-κB in response to inflammatory stimuli. Interestingly, the synergistic coactivation of PARP-1, p300, and also the Mediator complex was dependent on acetylation of PARP-1. Moreover, PARP-1 interacted with and is potentially deacetylated *in vivo* by class I HDACs. PARP-1-dependent transcriptional activation of NF-κB was negatively regulated by HDACs 1–3 *in vivo*. Together, these results support the hypothesis that acetylation of PARP-1 is important for its role as transcriptional coactivator and that the different physiological functions of PARP-1 might be in general regulated by post-translational modifications in a stimulus-dependent manner.

MATERIALS AND METHODS

Reagents—Recombinant TNFα was obtained from R&D Systems. Phorbol esters and mouse interferon-γ was purchased from Sigma. LPS (*Escherichia coli*, O26:B6) prepared by phenol extraction was purchased from Sigma and prepared as dispersed sonicate in endotoxin-free water before diluting to a final concentration in supplemented media. Nonfat dry milk was obtained from Migros (Switzerland). Nitrocellulose membranes were purchased from Osmonics Inc. Tosyl-activated Dynabeads were purchased from Dynal Biotech GmbH.

Plasmids—GST-PARP-1 and GST-p50 full-length expression vectors, CMV-PARP-1, and CMV-p300 expression vectors were described in Hassa *et al.* (8) and Covic *et al.* (16). The CMV expression vectors for the different Mediator subunits were generated by PCR or were a generous gift from Dr. L. Freedman (Merck Research Laboratories, West Point, NY). The CMV expression vectors for Myc-tagged HDACs 1–3 were created by PCR. The CMV expression vectors for FLAG or hemagglutinin-tagged HDAC 4–6 were kind gifts from Dr. S. L. Schreiber (Harvard University, Cambridge, MA). The expression vectors for CDK8/cyclin C and subunits of TFIID and TFIIF were kind gifts from Dr. M. Otsuka (University of Tokyo, Japan), Dr. Z. F. Burton (Michigan State University, East Lansing, MI), and Dr. G. Napolitano (University of Naples "Frederico II," Naples, Italy). NF-κB-dependent luciferase reporter constructs for MIP-2 (MIP-2 (−531/wt and MIP-2 (-531/mutκB)-Luc) and iNOS (iNOS (1485/+31wt)-Luc and iNOS(1485/+31-mutκB)-Luc) were generous gifts from Dr. H.-J. Kwon, (Yonsei University, Seoul, Korea) and Dr. M. A. Perrella (Harvard School of Public Health, Boston, MA) and are described in Perrella *et al.* (23) and Kim *et al.* (24). pphRSV-nt-β-galactosidase is described in Hassa *et al.* (20). The baculovirus for CDK8 and cyclin C was a generous gift from Dr. R. Pinhero (University of Guelph, Ontario, Canada) (25). The baculovirus for human p300 and mouse CBP was a generous gift from Dr. W. L. Kraus (Cornell University, New York) and Dr. D. Thanos (Institute of Molecular Biology and Genetics, Alexander Fleming Biomedical Sciences Research Center, Athens, Greece). The baculovirus for PARP-1 full-length and different domains were created by PCR using the PAK-8/9 system (BD Biosciences). GST-fusion expression vectors for different domains of PARP-1 were created by PCR. The PARP-1 mutant constructs were obtained by site-directed mutagenesis and confirmed by sequencing.

Mice Breeding Conditions, Cell Culture, and Transient Transfections—129S/EV-PARP-1(+/+) and 129S/EV-PARP-1(−/−) mice were bred under specified pathogen-free conditions. The initial 129S/EV-PARP-1(+/+) and 129S/EV-PARP-1(−/−) mice breeding pairs were a kind gift from Dr. Z. Q. Wang (26) (International Agency for Research on Cancer, Lyon, France). Primary macrophage cells were isolated from fresh littermates of 129S/EV-PARP-1(+/+) and 129S/EV-PARP-1(−/−) mice (20, 26) according to an isolation procedure described in Petrilli *et al.* (19), and human monocyte/macrophage-like THP-1 cells and Jurkat-T cells were grown in Hepes-buffered RPMI-Glutamax-I (Invitrogen) containing 10% fetal calf serum U. S.-certified (Invitrogen) and supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin (Invitrogen), 1 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, and minimum Eagle's medium-nonessential amino acids. Primary mouse fibroblast cells were grown in Hepes-buffered Dulbecco's modified Eagle's medium-Glutamax-I (Invitrogen) containing 4.5 g/liter glucose, 10% fetal calf serum U. S.-certified (Invitrogen) and supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin (Invitrogen), 1 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, and minimum Eagle's medium-nonessential amino acids. HEK293 cells were grown in Hepes-buffered Dulbecco's modified Eagle's medium-Glutamax-I (Invitrogen) containing 4.5 g/liter glucose and 10% fetal calf serum U. S.-certified (Invitrogen) and supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin (Invitrogen), and minimum Eagle's medium-nonessential amino acids. For luciferase reporter assays, cells were grown in 24- or 48-well dishes. Cells were transfected using calcium phosphate or polyethyleneimine procedures (as described in Refs. 27–30) except that primary cells were grown for 12 h in RPMI medium containing 2% fetal calf serum before stimulation with TNFα or LPS/IFNγ. The amount of DNA indicated in the figure legends was calculated for 10 ml of medium. Total amounts of DNA and equal molar ratios of promoters were kept constant in all set-ups by using empty vectors. For primary cells only cell passages 1–4 were used for transfection experiments. Because of differences in transfection efficiencies, an expression plasmid of β-galactosidase (pph-RSV-nt-β-Gal) was co-transfected as a transfection efficiency control, and luciferase activities were normalized based on β-galactosidase activity. Luciferase activity was measured as previously described in Hottiger *et al.* (27). For further experimental procedures see the supplemental material.

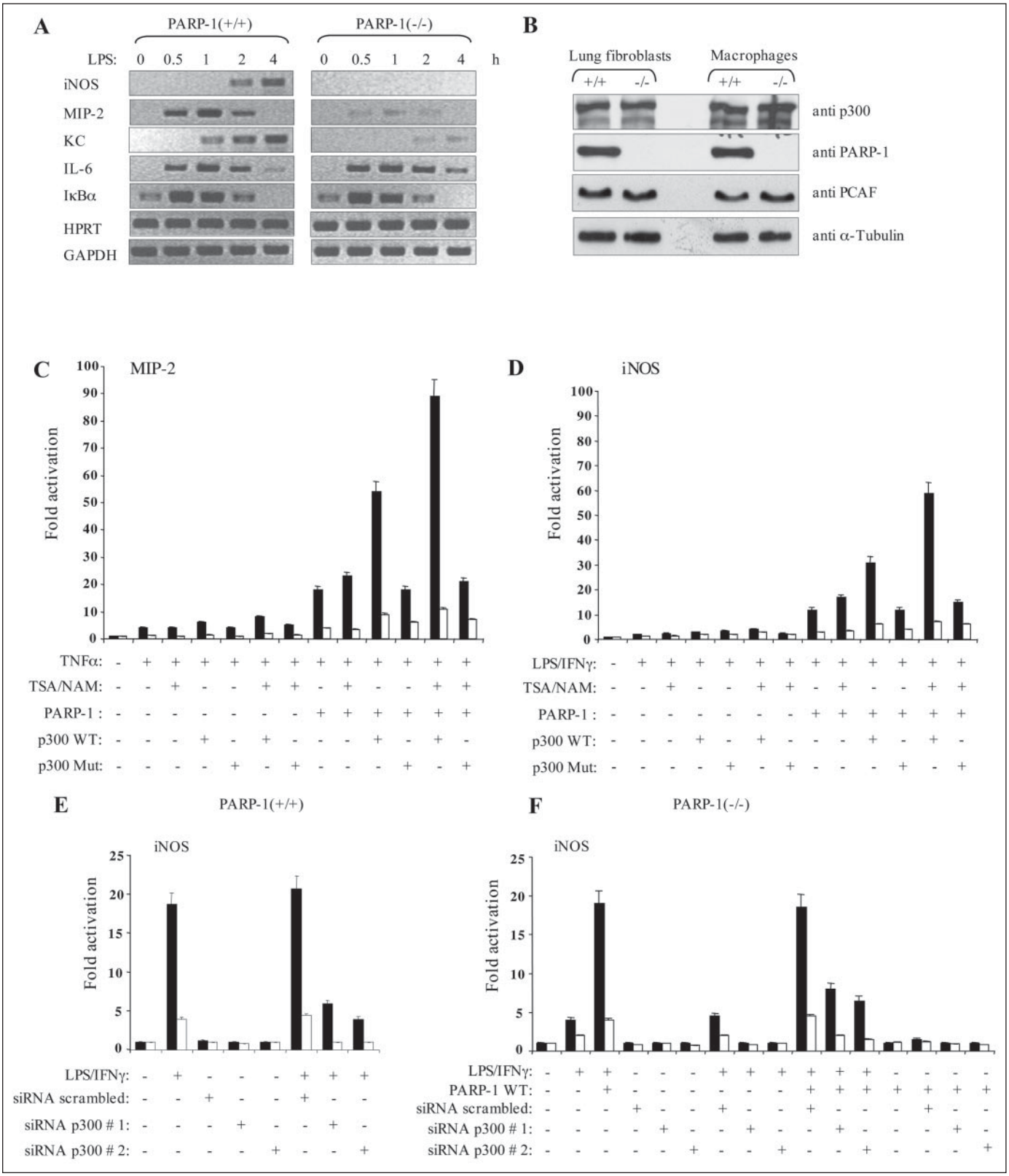

FIGURE 1. **PARP-1 is acetylated upon stimulation *in vivo* and requires the enzymatic activity of p300 for full NF-κB-dependent transcriptional activity.** *A*, primary PARP-1(+/+) or PARP-1(−/−) macrophages were treated with LPS (0.1 μg/ml) as indicated, and the expression of hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and NF-κB-dependent genes KC, MIP-2, iNOS, IκBα and interleukin-6 (*IL-6*) was assessed by reverse transcription-PCR. *B*, equal amounts of total cell extracts from primary PARP-1(+/+) and PARP-1(−/−) lung fibroblasts or primary PARP-1(+/+) and PARP-1(−/−) macrophages were resolved by SDS-PAGE followed by immunoblot analysis using anti-p300, anti-PARP-1, anti-PCAF, or anti- α-tubulin antibodies. *C* and *D*, primary PARP-1(−/−) macrophages were cotransfected with RSV-nt-β-Gal (300 ng), expression vectors for PARP-1 (2 μg), and wild type (*WT*) or an enzymatic mutant (*Mut*) of p300 (2 μg) along with a luciferase reporter under the control of the endogenous MIP-2 (1 μg) or iNOS (3 μg) promoters; cells were subsequently treated for 4 h with TNFα (10 ng/ml) or LPS/IFNγ (0.05 μg/ml/100 units) in the simultaneous presence or absence of low doses of deacetylase inhibitors (10 nM TSA/200 μM NAM). Cells were harvested 24 h after transfection, and NF-κB-dependent gene expression was determined. The indicated activation was determined by the ratio of the relative luciferase activity measured for the promoters containing wild type κB sites (*black bars*) or mutκB sites (*white bars*) after stimulation. The ratio

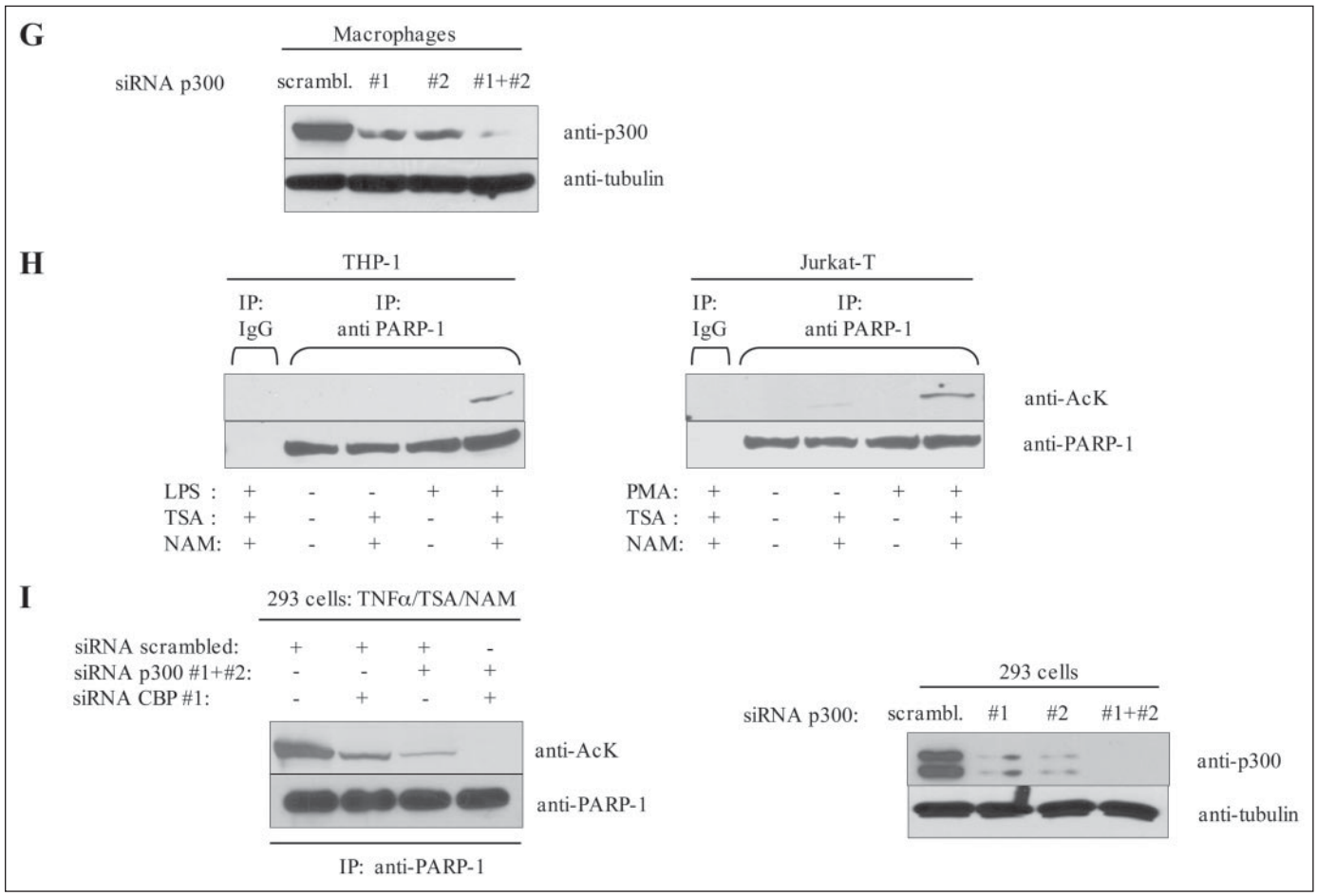

FIGURE 1—*continued*

RESULTS

PARP-1 Requires the Enzymatic Activity of p300 for Full NF-κB-dependent Transcriptional Activity and Is Acetylated in Vivo by p300/CBP upon Stimulation—Because the coactivator activity of PARP-1 for NF-κB-dependent gene expression is dependent on the stimuli and cell type (17), we first tested which NF-κB-dependent genes are impaired in freshly isolated primary PARP-1(−/−) macrophages. Primary PARP-1(+/+) or PARP-1(−/−) macrophages were treated with LPS as indicated, and the expression of NF-κB-dependent genes was assessed by reverse transcription-PCR (Fig. 1*A*). The experiments revealed that LPS-induced levels of KC, MIP-2, and iNOS were impaired in PARP-1(−/−) cells (Fig. 1*A*). The expression of IκBα and interleukin-6 (*IL-6*) was not reduced (Fig. 1*A*), indicating that only a subset of NF-κB-dependent genes requires PARP-1 for gene induction in these cells. Similar results were obtained when cells were stimulated with TNFα (data not shown). To confirm that the expression levels of histone acetyltransferases are not reduced in primary PARP-1 (−/−) cells, we tested the protein levels of p300 and PCAF by immunoblot analysis using anti-PARP-1, anti-p300, or anti-PCAF antibodies. The endogenous protein levels of p300 and PCAF were not impaired in freshly isolated primary PARP-1(−/−) lung fibroblast or macrophage cells (Fig. 1*B*).

PARP-1 and p300/CBP were shown to form a complex and function synergistically to enhance NF-κB-mediated gene expression (8). To test whether the synergistic coactivation of NF-κB-mediated transactivation by PARP-1 and p300/CBP might require the enzymatic activity of p300/CBP, we transfected PARP-1(−/−) cells with expression vectors for PARP-1 and wild type or an enzymatic mutant of p300 along with a luciferase reporter under the control of the endogenous MIP-2 or iNOS promoters, shown to be PARP-1-dependent. Cells were subsequently treated with TNFα or LPS/IFNγ in the simultaneous presence or absence of low doses of deacetylase inhibitors (TSA and nicotinamide (NAM)) (Fig. 1, *C* and *D*). Coexpression of wild type p300 with PARP-1 in PARP-1(−/−) cells resulted in a highly synergistic enhancement of transcription regulated from both MIP-2 and iNOS promoters upon stimulation (Fig. 1, *C* and *D*). However, the cooperativity between p300 and PARP-1 was severely impaired when an enzymatic mutant of p300

obtained for untreated cells was arbitrarily set to 1. *Error bars* indicate S.E. of three independent experiments. *E* and *F*, primary PARP-1(+/+) and PARP-1(−/−) macrophages were repeatedly transfected with scrambled- or p300-siRNA targeting vector DNA (30 μg) as indicated and then transfected and treated as indicated and described in *D*. The indicated activation was determined as described in *D*. *G*, equal amounts of total cell extracts from primary macrophages repeatedly transfected with scrambled- or p300-siRNA targeting vector DNA (30 μg) as indicated were resolved by SDS-PAGE followed by immunoblot analysis using anti-p300 or anti- α-tubulin antibodies. *H*, THP-1 or Jurkat-T cells were treated as indicated with LPS (0.2 μg/ml) or phorbol ester (10 nM) and simultaneously with or without deacetylase inhibitors (100 nM TSA/400 μM NAM) for 30 min. PARP-1 was immunoprecipitated (*IP*) under high salt conditions from nuclear extracts, and the presence of acetylated forms of PARP-1 was subsequently tested by immunoblot analysis using anti-AcK or anti-PARP-1 antibodies. *PMA*, phorbol 12-myristate 13-acetate. *I*, *left panel*, 293 cells were repeatedly transfected with scrambled-, p300-, or CBP-siRNA targeting vector DNA (30 μg) and treated as indicated. PARP-1 was immunoprecipitated under high salt conditions from nuclear extracts, and the presence of acetylated forms of PARP-1 was subsequently tested by immunoblot analysis using anti-AcK or anti-PARP-1 antibodies. *Right panel*, equal amounts of total cell extracts from 293 cells repeatedly transfected with scrambled- or p300-siRNA targeting vector DNA (30 μg) as indicated were resolved by SDS-PAGE followed by immunoblot analysis using anti-p300 or anti- α-tubulin antibodies.

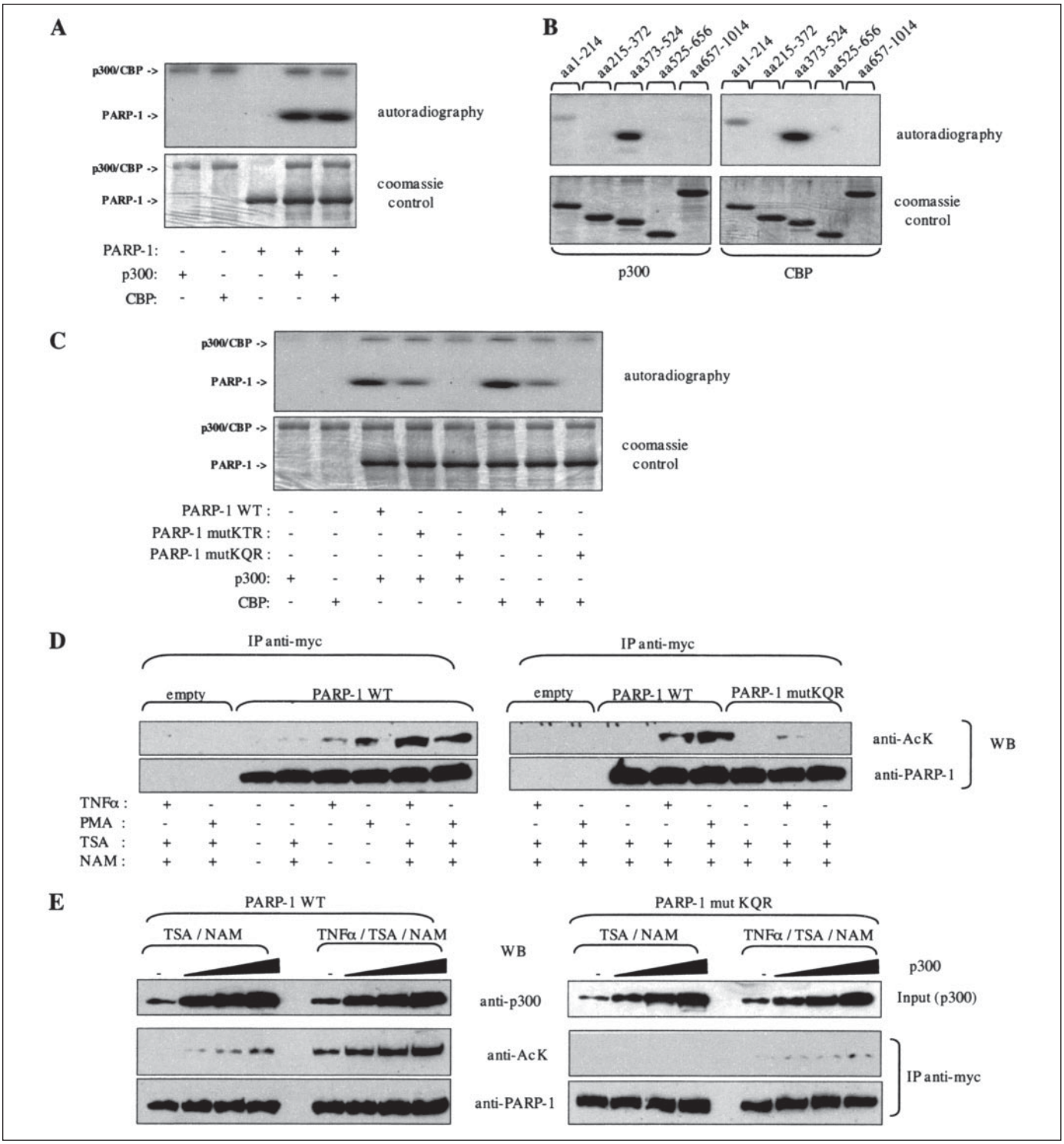

FIGURE 2. **PARP-1 is acetylated *in vitro* and *in vivo* at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 by p300/CBP upon stimulation.** *A*, recombinant purified full-length PARP-1 was incubated with recombinant purified full-length p300 or CBP in the presence of radioactive labeled acetyl-CoA and resolved by SDS-PAGE followed by autoradiography. *B*, baculo-purified PARP-1 fragments corresponding to amino acids (*aa*) 1–214, 215–372, 373–525, 525–656, or 657–1014 were incubated with recombinant purified full-length p300 or CBP in the presence of radioactive labeled acetyl-CoA and resolved by SDS-PAGE followed by autoradiography. *C*, recombinant purified full-length PARP-1 wild type and two mutant forms of PARP-1; mutKTR (K498R/K521R/K524R) and mutKQR (K498R/K505R/K508R/K521R/K524R) were incubated with recombinant purified full-length p300 or CBP in the presence of radioactive labeled acetyl-CoA and resolved by SDS-PAGE followed by autoradiography. *D*, Myc-tagged PARP-1 wild type (*WT*) or mutant mutKQR were overexpressed in 293 cells and treated as indicated with TNFα (10 ng/ml) and/or deacetylase inhibitors (100 nM TSA/400 μM NAM) for 30 min. Myc-tagged PARP-1 wild type or mutant mutKQR was then immunoprecipitated (*IP*) under high salt conditions from nuclear extracts of 293 cells, and the presence of acetylated forms of PARP-1 was subsequently tested by immunoblot analysis (*IB*) using anti-AcK or anti-PARP-1 antibodies. *E*, the same experiments were repeated in the presence of increasing amounts of overexpressed p300. Immunoblot of p300 input is shown in the *upper panels*

was coexpressed. The most striking differences between wild type and the enzymatic mutant of p300 were observed in the presence of low doses of deacetylase inhibitors (Fig. 1, *C* and *D*). The same transfection experiments with a reporter gene under the control of mutated κB sites revealed that the observed cooperative effect of p300 and PARP-1 was mainly NF-κB-specific (Fig. 1, *C* and *D*). To further confirm these

results, the same experiments were repeated in the presence of different p300-siRNA targeting vectors (Fig. 1, *E* and *F*). These experiments revealed that the presence of endogenous p300 is required for PARP-1-dependent coactivation of NF-κB-dependent gene expression (Fig. 1, *E* and *F*). Together these results indicate that the enzymatic activity of p300/CBP is required for NF-κB-dependent transactivation of extrachromosomal templates upon treatment with inflammatory stimuli.

p300 and CBP are known to modify a variety of proteins, such as histones and transcription factors (31). To investigate whether endogenous PARP-1 itself might be acetylated by p300/CBP *in vivo*, we immunoprecipitated endogenous PARP-1 under high salt conditions from nuclear extracts of THP-1 or Jurkat-T cells upon treatment with the indicated stimuli (LPS or TNFα) in the simultaneous presence or absence of deacetylase inhibitors (Fig. 1*H*, *left* and *right panels*). Possible acetylation of PARP-1 was analyzed by immunoblot analysis using an anti-AcK antibody. Endogenous PARP-1 was acetylated *in vivo* only in the presence of deacetylase inhibitors upon stimulation of these cells (Fig. 1*H*, *left* and *right panels*). Next we tested whether the observed acetylation of PARP-1 is dependent on p300/CBP. 293 cells were cotransfected with a control-siRNA vector and/or different siRNA targeting vectors for p300 or CBP. Endogenous PARP-1 was immunoprecipitated under high salt conditions from nuclear extracts of 293 cells upon treatment with TNFα in the presence of deacetylase inhibitors (Fig. 1*H*, *left* and *right panels*). These results revealed that endogenous PARP-1 was only acetylated *in vivo* upon stimulation in presence of endogenous p300 or CBP (Fig. 1*I*).

PARP-1 Is Acetylated by p300/CBP in Vitro and in Vivo at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524—Next, we tested whether PARP-1 can be acetylated by p300 or CBP *in vitro*. Recombinant-purified full-length PARP-1 was incubated with recombinant-purified full-length p300 or CBP in the presence of radioactive labeled acetyl-CoA and resolved by SDS-PAGE followed by autoradiography analysis (Fig. 2*A*). PARP-1 was strongly acetylated *in vitro* by p300 or CBP (Fig. 2*A*). To map the domains within PARP-1 subjected to acetylation by p300 and CBP, the same *in vitro* acetylation assay was repeated with different PARP-1 fragments corresponding to amino acids 1–214, 215–372, 373–525, 525–656, or 657–1014. These experiments revealed that p300 and CBP strongly acetylated the PARP-1 domain corresponding to amino acids 373–525 (Fig. 2*B*, *left* and *right panels*). Interestingly, a longer autoradiography exposure revealed that the domain corresponding to amino acids 1–214 of PARP-1 and to the small product of caspase cleaved PARP-1, was weakly acetylated by p300 or CBP (supplemental information and data not shown). The physiological relevance of this finding is currently under investigation. To identify the lysines acetylated by p300/CBP within PARP-1, recombinant purified full-length PARP-1 was acetylated *in vitro* by p300 full-length and analyzed by microcapillary reverse-phase high performance liquid chromatography nanoelectrospray tandem mass spectrometry. Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 were identified as strong candidates for acetylation. To confirm that these lysines are the main acceptor sites, full-length PARP-1 wild type or two PARP-1 mutant forms, mutKTR (K498R/K521R/K524R) and mutKQR (K498R/K505R/K508R/K521R/K524R) were incubated with p300 or CBP in the presence of radioactive labeled acetyl-CoA and resolved by SDS-PAGE followed by autoradiography analysis (Fig. 2*C*). These experiments confirmed that only the PARP-1 mutant harboring all five mutated lysines was no longer acetylated *in vitro* by p300 and CBP (Fig. 2*C*). To investigate whether these lysines are also the main acceptor sites for acetylation *in vivo* by p300, we immunoprecipitated Myc-tagged PARP-1 wild type or mutant mutKQR under high salt conditions from nuclear extracts of 293 cells upon treatment with the indicated stimuli in the presence or absence of deacetylase inhibitors (Fig. 2*D*). The presence of acetylated forms of PARP-1 was tested by immunoblot analysis using an anti-AcK antibody. PARP-1 was acetylated *in vivo* at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 in the presence of deacetylase inhibitors upon stimulation (Fig. 2*D*, also see the supplemental information). The same experiments performed in the presence of increasing amounts of overexpressed p300 suggested that these lysines are acetylated *in vivo* by p300 (Fig. 2*E*).

Acetylation of PARP-1 at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 Is Required in Vivo for Full NF-κB-dependent Transcriptional Activity—To directly test whether acetylation of PARP-1 is required for NF-κB-dependent transcriptional activity *in vivo*, we transfected PARP-1(−/−) cells with expression vectors for p300 and PARP-1 wild type or mutant mutKQR along with a luciferase reporter under the control of the endogenous MIP-2 or iNOS promoters. Cells were subsequently treated with the indicated stimuli (TNFα or LPS/IFNγ) in the simultaneous presence or absence of low doses of deacetylase inhibitors (Fig. 3, *A* and *B*). Coexpression of wild type p300 and wild type PARP-1 in PARP-1(−/−) cells caused a highly synergistic enhancement of transcription regulated from both MIP-2 and iNOS promoters upon stimulation (Fig. 3, *A* and *B*). However, the cooperativity between p300 and PARP-1 was severely impaired when the PARP-1 mutant mutKQR was coexpressed. The most striking differences between PARP-1 wild type and the mutant mutKQR were observed in the presence of low doses of deacetylase inhibitors. The same transfection experiments with a reporter gene under the control of mutated κB sites revealed that the observed induction was mainly NF-κB-specific (Fig. 3, *A* and *B*; and supplemental information).

Pavri *et al.* (32) have very recently shown that PARP-1 can associate *in vivo* with the Mediator. Therefore, we repeated the same transfection experiments in the presence of overexpressed subunits of the Mediator complex. Coexpression of PARP-1 wild type, p300, and Mediator subunits in PARP-1(−/−) cells caused a synergistic enhancement of NF-κB-dependent transcription, whereas no synergistic enhancement was observed when the mutant form of PARP-1, mutKQR, was coexpressed (Fig. 3, *C* and *D*), indicating that acetylation of PARP-1 is also required for the transcriptional cooperativity between p300/CBP, Mediator, and PARP-1 on these promoters.

PARP-1 Interacts in Vivo with the Mediator Complex and in Vitro Directly with the Mediator Subunits CDK8 and DRIP150—To further confirm these data and to investigate whether CDK8 or other subunits of the Mediator complex might directly interact with PARP-1, we first coexpressed tagged forms of different Mediator subunits in 293 cells (Fig. 4*A*). Immunoprecipitation experiments using an anti-PARP-1 antibody revealed that PARP-1 interacted with the whole Mediator complex (Fig. 4*A*). Next, we repeated these experiments with primary macrophages (Fig. 4*B*). These experiments revealed that endogenous PARP-1 could indeed interact with the endogenous Mediator complex under physiological conditions (Fig. 4*B*). DNA did not mediate the association of PARP-1 with the Mediator in the nucleus since the presence of ethidium bromide or DNase I did not affect PARP-1/Mediator interaction (data not shown). Surprisingly, whereas the interaction of PARP-1 with the core Mediator module was increased upon stimulation, the interaction of PARP-1 with CDK8 was decreased (Fig. 4, *A* and *B*). CDK8 is thought to act mainly as a repressor sub-module of the Mediator complex (32).

Because these results strongly suggested that PARP-1 would directly interact with at least one of these Mediator subunits, recombinant purified GST-PARP-1 full-length was bound to glutathione beads followed by incubation with *in vitro* translated and radioactive-labeled Mediator

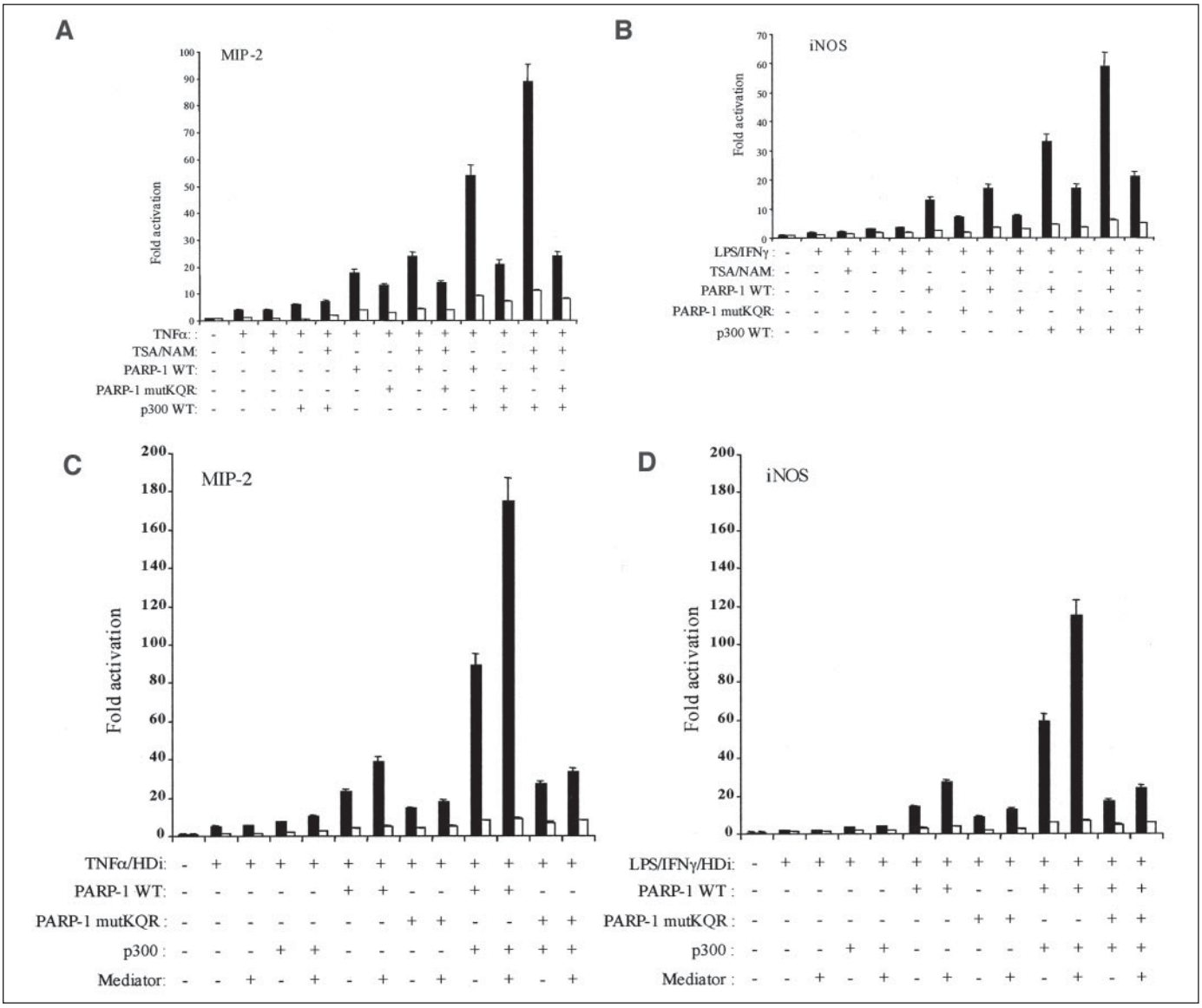

FIGURE 3. **Acetylation of PARP-1 is required for NF-κB-dependent transcription and for the transcriptional cooperativity between PARP-1, p300/CBP, and Mediator *in vivo*.** *A* and *B*, primary PARP-1(−/−) macrophages were cotransfected with RSV-nt-β-Gal (300 ng), expression vectors for p300 (2 μg), and PARP-1 wild type (*WT*) or mutant mutKQR (2 μg) along with a luciferase reporter under the control of the endogenous MIP-2 (1 μg) (*A*) or iNOS (3 μg) (*B*) promoters; cells were subsequently treated for 4 h with TNFα (10 ng/ml) or LPS/IFNγ (0.05 μg/ml/100 units) in the simultaneous presence or absence of low doses of deacetylase inhibitors (10 nM TSA/200 μM NAM). Cells were harvested 24 h after transfection, and NF-κB-dependent gene expression determined as described in Fig. 1*C*. *C* and *D*, primary PARP-1(−/−) macrophages were cotransfected with RSV-nt-β-Gal (300 ng), expression vectors for p300 (2 μg), MED-1 (2.5 μg), MED-7 (0.4 μg), MED-14 (2 μg), MED-15 (1.1 μg), MED-17 (0.75 μg), MED-23 (1.5 μg), MED-24 (1 μg), and PARP-1 wild type or mutant K498R/K505R/K508R/K521R/K524R ((*mutKQR*) (2 μg) along with a luciferase reporter under the control of the endogenous MIP-2 (1 μg) (*C*) or iNOS (3 μg) (*D*) promoters; cells were subsequently treated for 4 h with TNFα (10 ng/ml) or LPS/IFNγ (0.05 μg/ml/100 units) in the simultaneous presence of low doses of deacetylase inhibitors (*HDi*; 10 nM TSA/200 μM NAM). Cells were harvested 24 h after transfection and NF-κB-dependent gene expression was determined as described in Fig. 1*C*.

subunits as indicated in Fig. 4*C*. After extensive washes, bound proteins were resolved by SDS-PAGE followed by autoradiography analysis for Mediator subunits. PARP-1 directly bound to CDK8 and MED14, although to a low extent, but not to the other tested subunits (Fig. 4*C*). We next tested whether PARP-1 might also directly interact with other components of the RNA polymerase II machinery. Recombinant purified HMG(YI), CDK8, cyclin C, TFIIF/RAP74, TFIIF/RAP30, TATA box-binding protein (TBP), and TBP-associated factors fused to GST were bound to glutathione beads followed by incubation with recombinant baculo-purified PARP-1 (Fig. 4*D*, *left* and *right panels*). After extensive washes, bound proteins were resolved by SDS-PAGE followed by immunoblot analysis for PARP-1. PARP-1 was able to bind directly to HMG(YI), CDK8, and TFIIF/RAP74 but not to the other factors tested (Fig. 4*D*, *left* and *right panels*).

Acetylation of PARP-1 at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 Stabilizes the Interaction of PARP-1 with p50—To investigate whether acetylation of PARP-1 mechanistically influences protein-protein interactions, we first tested which of the interaction partners associates with the acetylated domain of PARP-1. Different domains of PARP-1 fused to GST were bound to glutathione beads and incubated with purified p300, PARP-1, CDK8/cyclin C, p65, p50, TFIIF/RAP74 or *in vitro* transcribed/translated and radioactive-labeled MED14 (Fig. 5, *A* and *B*). After extensive washes, bound proteins were resolved by SDS-PAGE followed by immunoblot analysis with the indicated antibodies (Fig. 5*A*) or autoradiography (Fig. 5*B*). p300, PARP-1, CDK8, p65, p50, TFIIF/RAP74, and MED14 bound to different domains of PARP-1 as schematically drawn in Fig. 5*C*. PARP-1 interacted with a region between amino acids 1 and 214 as well as between amino acids 465 and

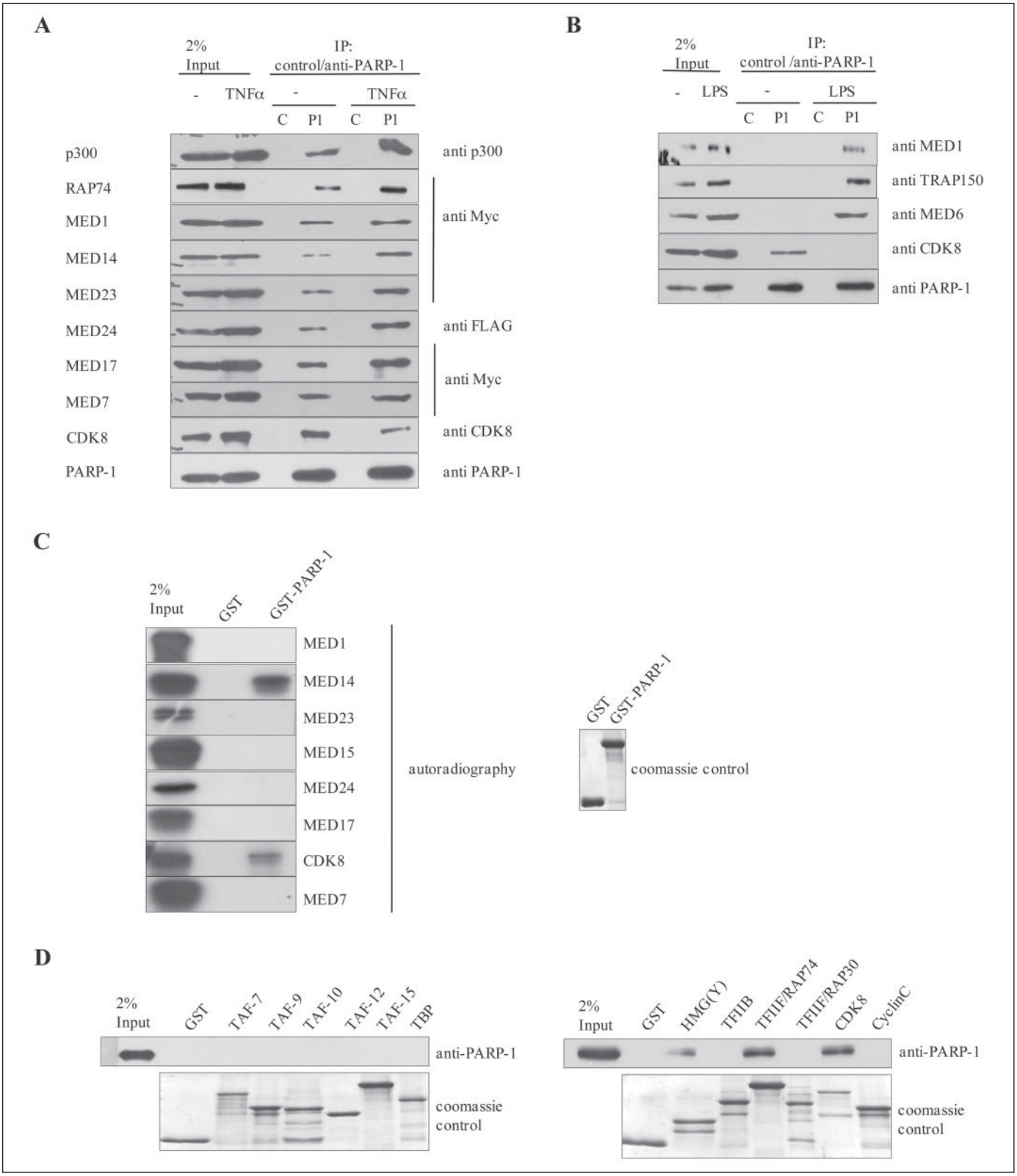

FIGURE 4. **PARP-1 interacts with the Mediator complex *in vivo* and directly with the Mediator subunits MED14 and CDK8 *in vitro*.** *A*, Myc- or FLAG-tagged Mediator subunits MED-1, MED-7, MED-14, MED-17, MED-23, and MED-24 were overexpressed in 293 cells and treated as indicated, and PARP-1 complexes were immunoprecipitated (*IP*) under physiological salt conditions from nuclear extracts using an anti-PARP-1 antibody and tested for PARP-1 and Myc- or FLAG-tagged Mediator subunits, p300, and CDK8 by immunoblot analysis using anti-PARP-1, anti-p300, anti-CDK8, anti-Myc, or anti-FLAG antibodies. *C*, control IgG; P1, anti-PARP-1. *B*, primary macrophages were treated as indicated with LPS (0.2 μg/ml) for 1 h. PARP-1 was immunoprecipitated under physiological salt conditions from nuclear extracts, and the presence of Mediator subunits was subsequently tested by immunoblot analysis using anti-MED1, anti-MED6 anti-TRAP150, anti-CDK8, or anti-PARP-1 antibodies. *C*, GST pull down assays under physiological salt conditions with PARP-1 full-length fused to GST (3 μg GST fusion protein) and the indicated *in vitro* transcribed/translated Mediator subunits. Bound proteins were resolved by SDS-PAGE followed by autoradiography. The Coomassie control gel is shown in the *left panel*. *D*, GST pull down assays under physiological salt conditions with the indicated general transcription factors and CDK8/cyclin C fused to GST (1–4 μg of GST fusion protein) and baculo-purified PARP-1 (0.5 μg input). Bound proteins were resolved by SDS-PAGE followed by immunoblot analysis using anti-PARP-1 antibodies (*upper panel*). The corresponding Coomassie control gel is shown in the *lower panel*. *TBP*, TATA box-binding protein.

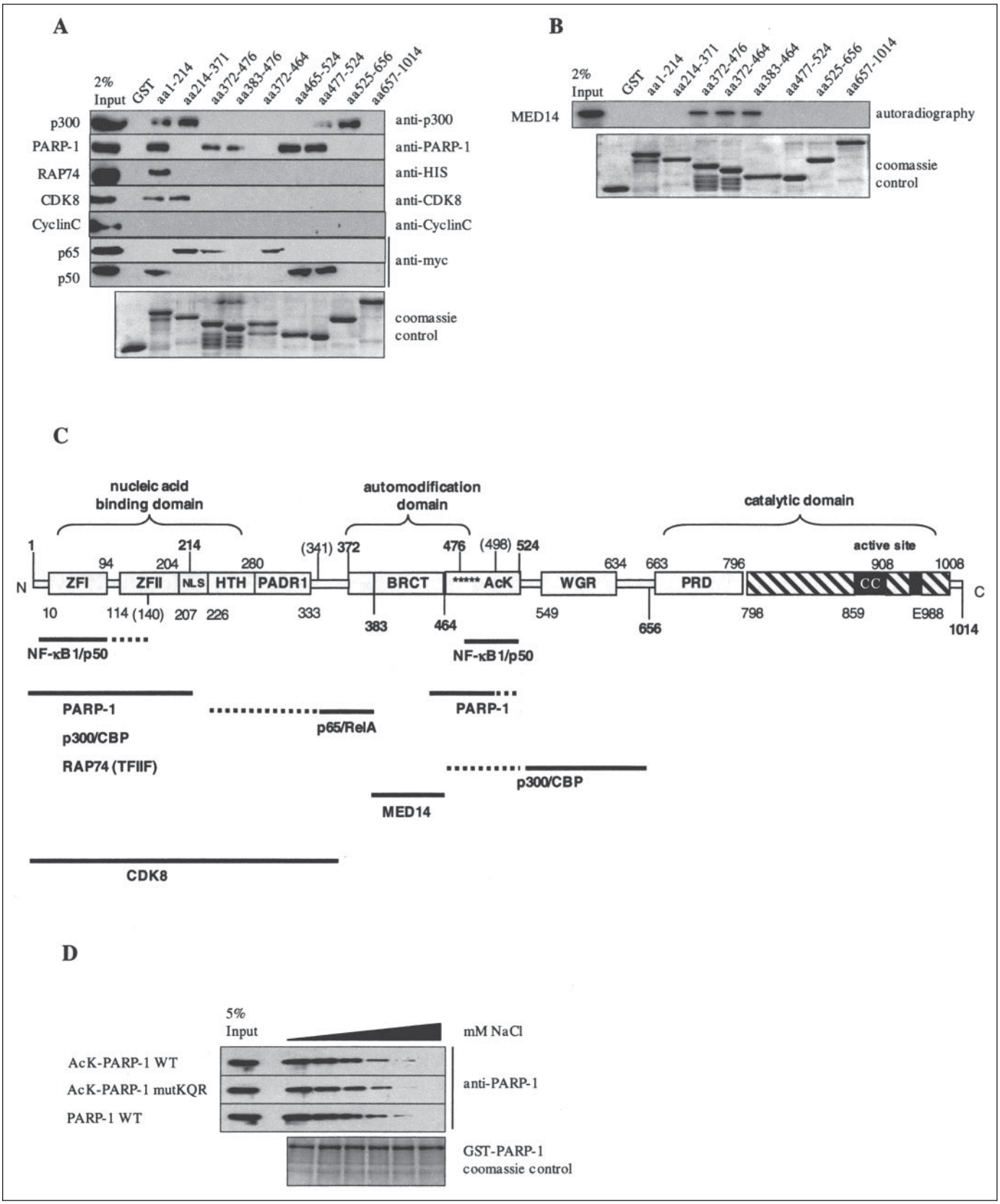

FIGURE 5. **Acetylation of PARP-1 stabilizes the interaction of PARP-1 with p50 but not p65 or PARP-1.** *A* and *B*, mapping of the interaction domains in PARP-1. Shown are GST pull down assays under high salt conditions with PARP-1 fragments fused to GST (1–3 μg of protein) as indicated and recombinant purified p300 (2 μg), PARP-1 (0.3 μg), RAP74 (1 μg), CDK8 (1 μg), cyclin C (0.5 μg), p65 (0.5 μg), and p50 (0.5 μg) (*A*) or *in vitro* transcribed/translated MED14 (*B*). Bound proteins were resolved by SDS-PAGE followed by immunoblot analysis with the corresponding antibodies (*A*) or autoradiography (*B*). The corresponding Coomassie control gel is shown in the *lower panels*. *aa*, amino acids. *C*, schematic picture of the PARP-1 interaction map: ZF I and ZFII, zinc finger 1 and 2; *NLS*, nuclear localization signal; *HTH*, helix turn helix domain, *BRCT*, C-terminal domain of a breast cancer susceptibility protein (BRCA); *WGR*, central WGR motif-containing domain;*PADR1*, PARP regulatory domain; *CC*, catalytic center. *D* and *E*, acetylation-dependent interaction of PARP-1 and p50. PARP-1 (*D*) or p50 (*E*) fused to GST (3 μg/2 μg) were incubated with *in vitro* acetylated or non-acetylated baculo-purified PARP-1 wild type (*WT*) or mutant form mutKQR (500 ng) in

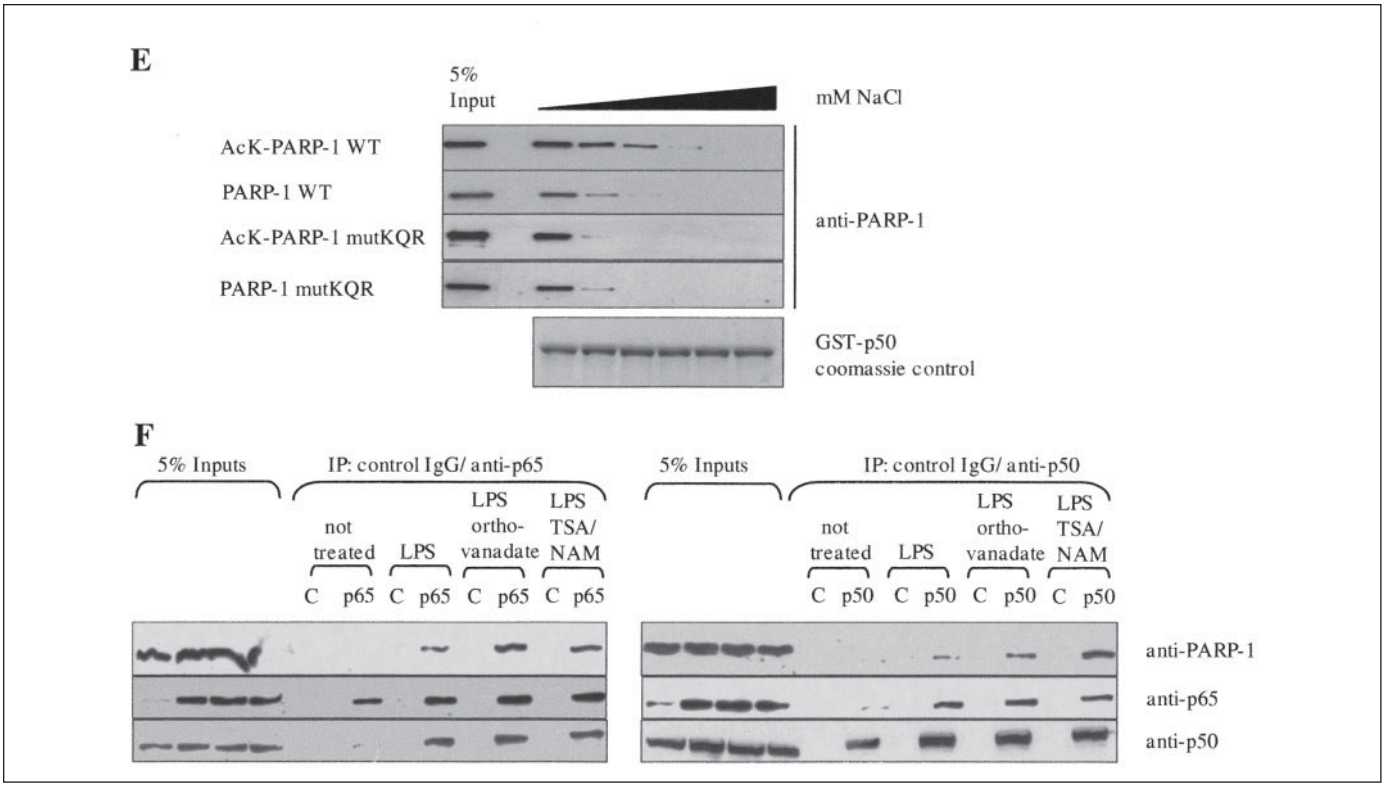

FIGURE 5—*continued*

524 (Fig. 5*C*). MED14 was the only tested protein that bound to the BRCT (carboxyl-terminal domain of a breast cancer susceptibility protein (BRCA)) domain of PARP-1. We have previously shown that p50 and p65 interact with a region between amino acids 341 and 531 of PARP-1 (20). In addition, p50 interacted also with a region between amino acids 1 and 140 containing the zinc finger I. Our more detailed interaction analysis suggests that p65 would interact with a region between amino acids 341 and 383 of PARP-1. p50 would interact with zinc finger I or zinc finger II and with a region between amino acids 477 and 524 (Fig. 5*C*). Together, these experiments revealed that only the interaction of PARP-1 with PARP-1 itself or p50 might be influenced by acetylation of PARP-1.

To test this hypothesis, recombinant purified PARP-1 or p50 fused to GST were bound to glutathione beads and incubated with non-acetylated or *in vitro* acetylated purified PARP-1 wild type and the mutant form mutKQR in the presence of increasing concentrations of NaCl as indicated (Fig. 5, *D* and *E*). After extensive washes, bound proteins were resolved by SDS-PAGE followed by immunoblot analysis for PARP-1 (Fig. 5, *D* and *E*). No significant differences between the acetylated or non-acetylated form of PARP-1 were observed for the dimerization or potential tetramerization of PARP-1 (Fig. 5*D*). Surprisingly, *in vitro* acetylated PARP-1 bound significantly better to p50 than did the non-acetylated PARP-1 (Fig. 5*E*). These results suggest that PARP-1 would dimerize or potentially tetramerize through the regions between amino acid 1 and 214 as well as between amino acid 465 and 497, whereas the region between amino acid 477 and 524 is required for acetylation-dependent interaction with p50 (Fig. 5*C*).

To confirm these *in vitro* data and to investigate whether acetylation would influence the stimuli-dependent complex formation between subunits of NF-κB and PARP-1 *in vivo*, we coimmunoprecipitated the NF-κB subunits p65 and p50 from nuclear extracts upon treatment of THP-1 with LPS in the presence or absence of deacetylase or phosphatase inhibitors (Fig. 5*F*, *left* and *right panels*) and tested the presence of PARP-1 by immunoblot analysis using anti-PARP-1 antibodies. Remarkably, PARP-1 bound significantly better to p65 when cells were simultaneously treated with LPS and phosphatase inhibitors (Fig. 5*F*, *left panel*), whereas p50 bound significantly better to PARP-1 upon simultaneous treatment with LPS and deacetylase inhibitors (Fig. 5*F*, *right panel*). No significant differences between deacetylase or phosphatase inhibitors were observed for the interaction between p50 and p65 (Fig. 5*F*, *left* and *right panels*). Taken together, these results suggest that phosphorylation might mainly enhance the interaction between p65 and PARP-1, whereas acetylation mainly enhances the interaction between p50 and PARP-1.

PARP-1 Interacts with Members of HDAC Class I and Is Potentially Deacetylated by HDACs 1–3 in Vivo—Because acetylation of proteins is known to be a reversible modification *in vivo*, we next tested whether PARP-1 might physically interact with HDACs *in vivo*. Because of the low quality of commercially available anti-HDAC antibodies, we decided to perform these experiments with overexpressed Myc- or FLAG-tagged HDACs. We coimmunoprecipitated PARP-1 complexes from nuclear extracts of untreated 293 cells overexpressing Myc-tagged versions of HDAC-1, HDAC-2, or HDAC-3 (Fig. 6*A*) and FLAG-tagged versions of HDAC-4, HDAC-5 or HDAC-6 (Fig. 6*B*) and tested the

presence of increasing concentrations of NaCl (125 mM NaCl, 25 mM potassium acetate to 225 mM NaCl, 25 mM potassium acetate) as indicated. Bound proteins were resolved by SDS-PAGE followed by immunoblot analysis using a anti-PARP-1 antibody. *F*, acetylation and stimuli-dependent complex formation of PARP-1 and subunits of NF-κB *in vivo*. NF-κB subunits p65 and p50 were coimmunoprecipitated (*IP*) from nuclear extracts upon treatment of THP-1 with LPS in the presence or absence of deacetylase or phosphatase inhibitors (*left* and *right panels*) and tested for the presence of p50, p65, and PARP-1 by immunoblot analysis using anti-p50, anti-p65, and anti-PARP-1 antibodies. *C*, control.

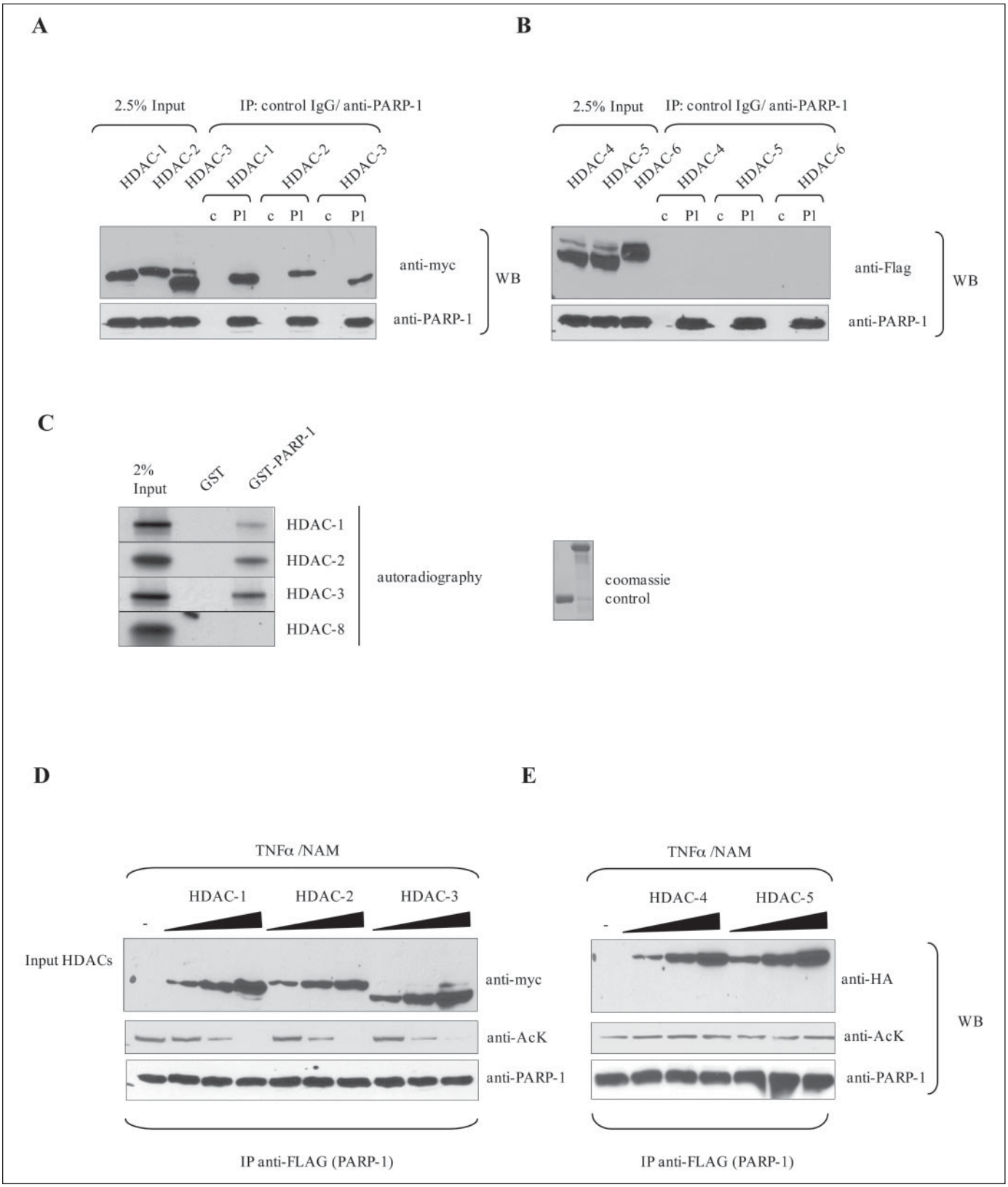

FIGURE 6. **PARP-1 interacts with members of HDAC class I and is potentially deacetylated by HDAC-1, HDAC-2, or HDAC-3 *in vivo*.** *A* and *B*, FLAG-tagged HDACs were overexpressed in 293 cells. PARP-1 complexes were co-immunoprecipitated (*IP*) from nuclear extracts of untreated 293 cells using an anti-PARP-1 (*P1*) antibody and subsequently tested for PARP-1 and Myc-tagged HDAC-1, HDAC-2, or HDAC-3 and FLAG-tagged HDAC-4, HDAC-5, or HDAC-6 by immunoblot (*WB*) analysis using anti-PARP-1, anti-Myc or anti-FLAG antibodies. *c*, control IgG. *C*, GST pull down assays under physiological salt conditions with PARP-1 fused to GST (4 μg of protein) and *in vitro* transcribed/translated HDAC-1, -2, -3, or -8. Bound proteins were resolved by SDS-PAGE followed by autoradiography (*left panel*). The corresponding Coomassie control gel is shown in the *right panel*. *D* and *E*, FLAG-tagged PARP-1 wild type was coexpressed with increasing amounts of Myc-tagged HDAC-1, HDAC-2, or HDAC-3 and hemagglutinin (*HA*)-tagged HDAC-4 or HDAC-5 in 293 cells. 24 h later cells were simultaneously treated with TNFα (10 ng/ml) and NAM (1 mM) for 30 min. FLAG-tagged PARP-1 was subsequently immunoprecipitated under high salt conditions from nuclear extracts of 293 cells, and the presence of acetylated forms of PARP-1 was tested by immunoblot analysis using anti-AcK or anti-FLAG antibodies.

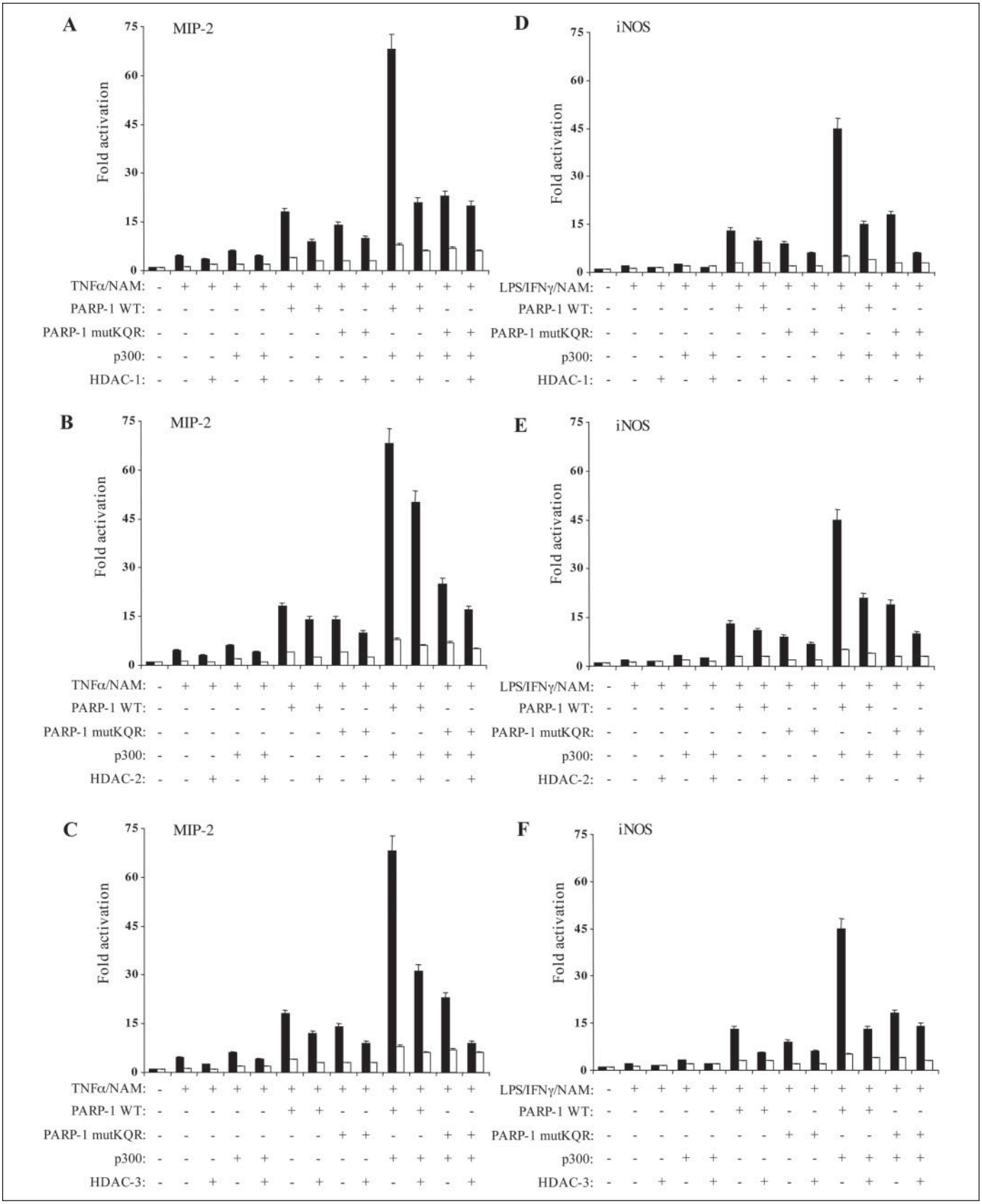

FIGURE 7. **The coactivator activity of PARP-1 is negatively regulated *in vivo* by HDAC-1 HDAC-2, or HDAC-3.** *A–F*, primary PARP-1(−/−) macrophages were cotransfected with expression vectors for RSV-nt-β-Gal (300 ng), PARP-1 wild type (*WT*) or mutant mutKQR (2 μg), p300 (2 μg), HDAC-1, HDAC-2, or HDAC-3 (2.5 μg each) along with a luciferase reporter under the control of the endogenous MIP-2 (1 μg) (*A–C*) or iNOS promoters (3 μg) (*D–F*); cells were subsequently treated for 4 h with TNFα (10 ng/ml) or LPS/IFNγ (0.05 μg/ml/100 units) in the simultaneous presence of low doses of deacetylase inhibitor (500 μM NAM). Cells were harvested 24 h after transfection, and NF-κB-dependent gene expression was determined as described in Fig. 1*C*.

presence of these HDACs by immunoblot analysis using anti-PARP-1, anti-Myc, or anti-FLAG antibodies. Interestingly, PARP-1 interacted with HDAC-1, HDAC-2, and HDAC-3 but not with HDAC-4, HDAC-5, or HDAC-6 (Fig. 6, *A* and *B*). DNA did not mediate the association of PARP-1 with HDACs since the presence of ethidium bromide or DNase I did not affect the interaction (data not shown). Because these results suggested that PARP-1 would directly interact with at least one of these HDACs, GST-PARP-1 full-length was bound to glutathione beads and incubated with *in vitro* translated and radioactive-labeled HDAC class I members HDAC-1, HDAC-2, HDAC-3, or HDAC-8 (Fig. 6*C*). After extensive washes, bound proteins were resolved by SDS-PAGE followed by autoradiography analysis for HDACs. PARP-1 bound directly to HDAC-1, HDAC-2, and HDAC-3 but not to HDAC-8 (Fig. 6*C*).

To investigate whether PARP-1 might be deacetylated by HDAC-1, HDAC-2, or HDAC-3 *in vivo*, FLAG-tagged PARP-1 wild type was coexpressed with increasing amounts of Myc-tagged HDAC-1, HDAC-2, or HDAC-3 (Fig. 6*D*) and hemagglutinin-tagged HDAC-4 or HDAC-5 in 293 cells (Fig. 6*E*). FLAG-tagged PARP-1 was immunoprecipitated from nuclear extracts under high salt conditions upon treatment of cells with TNFα and NAM. The presence of acetylated forms of PARP-1 was tested by immunoblot analysis using anti-AcK or anti-FLAG antibodies. These experiments revealed that PARP-1 might be deacetylated *in vivo* by HDAC-1, HDAC-2, or HDAC-3 (Fig. 6, *D* and *E*).

PARP-1-dependent Transcriptional Activation of NF-κB Seems to Be Negatively Regulated by HDACs 1–3 in Vivo—Next, we tested which HDAC has the strongest influence on the transcriptional coactivator activity of PARP-1 and might therefore also act functionally as a deacetylase for PARP-1 *in vivo*. PARP-1(−/−) cells were cotransfected with expression vectors for PARP-1 wild type or PARP-1 mutant form; that is, mutKQR, p300, HDAC-1, HDAC-2, or HDAC-3 along with a luciferase reporter under the control of the endogenous MIP-2 (Fig. 7, *A–C*) or iNOS promoters (Fig. 7, *D–F*). Cells were simultaneously treated with the indicated stimuli (TNFα or LPS/IFNγ) and low doses of deacetylase inhibitors (Fig. 7, *A–F*). Coexpression of p300 with HDAC-1, HDAC-2, or HDAC-3 in the absence of PARP-1 resulted in a reduced activation of NF-κB-dependent transcriptional activation (Fig. 7, *A–F*). No significant differences between HDAC-1, HDAC-2, or HDAC-3 were observed in the absence of PARP-1 for either MIP-2 or iNOS promoters (Fig. 7, *A–F*), indicating that the residual activation of NF-κB in PARP-1(−/−) cells in the absence of PARP-1 is equally repressed by HDAC-1, HDAC-2, or HDAC-3. Similar results were obtained in presence of PARP-1 wild type, although the repression by HDAC-2 was weaker when compared with HDAC-1 and HDAC-3 (Fig. 7, *A–F*). Remarkably, the repression by HDAC-1, HDAC-2, or HDAC-3 was strongly reduced when HDAC-1 HDAC-2, or HDAC-3 was coexpressed with PARP-1 mutant mutKQR (Fig. 7, *A–F*). Together these results suggest that HDAC-1, HDAC-2, and HDAC-3 might repress NF-κB-dependent transcription in part through deacetylation of PARP-1.

DISCUSSION

Growing experimental evidence suggests that PARP-1 can function as a promoter-specific coactivator (17). PARP-1 was also identified as an interaction partner not only of NF-κB but also of several sequence-specific transcription factors and cofactors including E2F1, Oct-1, and PC3/topoisomerase-I (17, 33) and has been shown to increase the transcriptional activity of these transcription factors (17). In 1997 Meisterernst *et al.* (34) identified human PARP-1 as one active component of the upstream stimulatory activity (USA)-derived positive cofactor complex PC-1 (34). The crude precursor human USA fraction consists of PC1/PARP-1, PC2/Mediator-like complex, PC3/topoisomerase-I, PC4/single-stranded DNA-binding protein, PC52, PC6, and HMG2 (34, 35). Earlier studies showed that full activation of transcription by NF-κB, Sp1, and Oct-1 in cell-free systems required a crude precursor USA coactivator fraction in addition to general transcription factors (36). Because PC1/PARP-1, PC3, and PC4 are all sequence-unspecific DNA-binding proteins, it was suggested that PC1/PARP-1 provide together with the other USA-derived positive cofactors PC3, PC4, PC52, and HMG2 a mainly structural/architectural role in assembling and stabilizing the pre-initiation complex by affecting the accessibility of RNA polymerase II to chromatin (17, 34, 35, 37).

The aim of this study was to investigate whether the coactivator activity of PARP-1 might be regulated by post-translational modifications such as acetylation. We provide both biochemical and functional evidence that acetylation of PARP-1 is required for its NF-κB coactivator activity.

PARP-1 Requires the Enzymatic Activity of p300 for Full NF-κB-dependent Transcriptional Activity—Several reports demonstrated that coactivator activity of PARP-1 for NF-κB-dependent gene expression seems to be dependent on the stimuli and cell type (17). We observed an impaired expression in primary PARP-1(−/−) macrophages of iNOS, KC, and MIP-2 genes but not of IκBα and IL-6 upon stimulation with TNFα or LPS/IFNγ, indicating that PARP-1 acts in a promoter-specific manner, similar to other coactivators of NF-κB such as CARM1 (16). PARP-1 and p300/CBP were shown to form a complex and function synergistically to enhance NF-κB-mediated gene expression (8). Moreover, we recently provided evidence that the enzymatic activities of p300/CBP and protein arginine methyltransferase CARM1 are required for NF-κB-dependent gene expression *in vivo* (16). NF-κB-dependent reporter gene analysis using the PARP-1-dependent MIP-2 and iNOS promoters revealed that the cooperativity between p300 and PARP-1 was severely impaired when an enzymatic mutant of p300 was coexpressed, indicating that the enzymatic activity of p300/CBP is required for NF-κB-dependent transactivation of PARP-1-dependent promoters upon treatment with inflammatory stimuli.

PARP-1 Is Acetylated in Vivo at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 by p300/CBP upon Stimulation—Growing experimental evidence has accumulated that NF-κB-dependent gene expression is also regulated by post-translational modifications such acetylation (22). This acetylation-dependent regulation was shown to occur at multiple levels (38). Acetylation of histones regulates the NF-κB-dependent gene accessibility (22). Moreover, direct acetylation of the NF-κB subunits p65 and p50 was shown to regulate transcriptional activation of NF-κB (39–41). Finally, other acetylation events were suggested to temporarily modulate the duration of NF-κB presence in the nucleus and DNA binding activity as well as protein-protein interactions with several cofactors involved in the transcriptional activity of NF-κB (22, 38). It was recently reported that the USA-derived positive cofactor PC4 is specifically acetylated by p300, thereby stimulating its double-stranded DNA binding activity which correlates with its coactivation activity (42, 43). Here we show that PARP-1 is acetylated *in vitro* and *in vivo* by p300 and CBP. Combined analysis by microcapillary reverse-phase high performance liquid chromatography nanoelectrospray tandem mass spectrometry and coexpression of p300/CBP and PARP-1 wild type or mutant revealed that PARP-1 is acetylated *in vivo* at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 by p300/CBP upon stimulation. *In vitro* acetylation experiments using purified recombinant PARP-1 wild type or mutant forms supported these *in vivo* observations.

Acetylation of PARP-1 at Lys-498, Lys-505, Lys-508, Lys-521, and Lys-524 Is Required for NF-κB-dependent Transcriptional Activity in Vivo—The importance of PARP-1 acetylation for NF-κB-dependent transcription is supported by complementation experiments in primary PARP-

1(−/−) macrophages using the PARP-1 mutant mutKQR (K498R/K505R/K508R/K521R/K524R). Full synergistic enhancement of transcription regulated from the MIP-2 and iNOS promoters was obtained only when PARP-1 wild type was coexpressed with p300 in PARP-1(−/−) macrophages. The observed residual cooperative effects in the presence of mutated κB sites on the promoter most likely reflect the coactivator activity of PARP-1 on other sequence-specific transcription factors such as activator protein-1, Sp-1, Oct-1, interferon regulatory factors, or signal transducers and activators of transcription (STATs), which are also important for full activity of NF-κB on these promoters (24, 44–46). Several recent reports provided evidence that PARP-1 might also act as a coactivator of AP-1 and STATs (47–49). Interestingly, the observed differences between PARP-1 wild type and mutant mutKQR regarding the co-operativity of p300 and PARP-1 were less significant in experiments using reporter plasmids with mutated κB sites, suggesting that acetylation of PARP-1 might be mainly required for the NF-κB-dependent promoter activity. However, it remains to be further investigated whether acetylation of PARP-1 could also strongly influence other sequence-specific transcription factors or cofactors under certain conditions. Remarkably, coexpression of PARP-1 wild type or mutant mutKQR with p300 and Mediator subunits in PARP-1(−/−) macrophages demonstrated that acetylation of PARP-1 is also required for the transcriptional cooperativity between p300/CBP, Mediator, and PARP-1 on these promoters. Recent reports showed that ARC/Mediator interacts with the transactivation domain of p65 and enhances chromatin-dependent transcriptional activation by p65 *in vitro* (50). The Mediator complex is thought to provide the penultimate step in the activation process by bridging a given activator to RNA polymerase II and forming a scaffold onto which RNA polymerase II and general transcription factors can assemble and initiate transcription (51). Based on the multistep interaction model of transcriptional activation proposed by Roeder and co-workers (51), it was suggested that PC1/PARP-1 might facilitate together with other structural/architectural positive cofactors the co-operative interactions between sequence-specific activators and different coactivator complexes such as p300/CBP and Mediator, thereby providing an architectural function in stabilizing the pre-initiation complex (17). In our current studies we provided evidence that PARP-1 interacts *in vivo* with the Mediator complex under physiological conditions. Furthermore, PARP-1 directly interacted *in vitro* with the Mediator subunits MED14 and CDK8 as well as the TFIIF subunit RAP74 but not with TFIIB, TATA box-binding protein (TBP) and the tested TBP-associated factors. These results are consistent with the observation of Meisterernst *et al.* (34) that in "*in vitro* transcription" assays, PARP-1 is only required during assembly of RNA polymerase II and general transcription factors on preformed TFIID-TFIIA-DNA complexes (34). In these studies PARP-1 was unable to stimulate the formation of a TFIID-promoter complex. In addition, PARP-1 stimulated transcription only when added before the complete pre-initiation complex was formed, which implies that PARP-1 might only function during assembly of the pre-initiation complex (34).

Whether acetylation of PARP-1 might regulate the PARP-1 activity at this level in the context of chromatin remains to be investigated. However, it seems unlikely since the Mediator subunits DRIP150 and CDK8 did not bind to the acetylated domain in PARP-1. Surprisingly, acetylation of PARP-1 regulates the stimuli-dependent interaction of PARP-1 with NF-κB1 subunit p50 but not p65. Because the acetylation mutant mutKQR of PARP-1 could still partially coactivate NF-κB, acetylation of PARP-1 might be mechanistically required for the stabilization of preformed PARP-1 containing transcriptional coactivator-cofactor complexes.

PARP-1-dependent Transcriptional Activation of NF-κB Seems to Be Negatively Regulated in Vivo by HDACs 1–3—Overexpression of HDAC-1, HDAC-2, and HDAC-3 was shown to repress NF-κB-dependent transcription upon treatment with inflammatory stimuli (22, 38, 41). HDAC-3 was shown to be required for the deacetylation of p65 (52). HDAC-1, HDAC-2, and HDAC-3 interact directly with several proteins involved in the NF-κB signaling pathway, including NF-κB itself (22, 52, 53). Interestingly, PARP-1 formed a complex with HDAC-1, HDAC-2, or HDAC-3 in the nucleus but not with HDAC-4, HDAC-5, or HDAC-6. The direct interaction *in vitro* between PARP-1 and HDACs 1–3 was weak and might be very transient or regulated *in vivo* by other unidentified post-translational modifications. Subsequent *in vivo* deacetylation experiments with increasing amounts of overexpressed HDACs suggested that deacetylation of PARP-1 *in vivo* might be mediated by HDAC-1, HDAC-2, or HDAC-3. Transient reporter assays revealed that the PARP-1-dependent transcriptional activity of NF-κB is negatively regulated *in vivo* by HDAC-1, HDAC-2, or HDAC-3. Because transiently transfected plasmids seem not to be properly chromatinized, nucleosomal histones are unlikely to represent the only relevant substrates whose lysine acetylation is required for NF-κB-dependent transcription. Remarkably, when HDAC-1, HDAC-2, or HDAC-3 was co-expressed with PARP-1 mutant mutKQR, no significant additional promoter-specific decrease in NF-κB-dependent transcription was obtained. These results suggest that HDAC-1, HDAC-2, and HDAC-3 might deacetylate promoter-bound PARP-1 or other unidentified cofactors or coactivators dependent on acetylation of PARP-1.

Interestingly, several recent reports demonstrated that inhibition of HDACs could selectively suppress transcription in a gene-specific manner by inducing an elongational arrest and/or premature termination between exons 1 and 2 (54–57). Thus, in the context of chromatin, it might be that for at least a subset of NF-κB-dependent genes, deacetylation of NF-κB itself or of coactivators involved in the nuclear activity of NF-κB, such as PARP-1, could be required for transcriptional elongation or re-initiation. For at least a subset of PARP-1-dependent NF-κB target genes, both the enzymatic activities of p300 and acetylation of PARP-1 are required for transcriptional activity. However, the exact molecular mechanism by which acetylation of PARP-1 regulates the coactivator activity of PARP-1 in the context of chromatin remains to be investigated. In addition, we cannot exclude the possibility that other histone acetyltransferases might acetylate PARP-1 on other lysine residues and thereby influence its activities. Whether p65 or other sequence-specific transcription factors known to cooperate with NF-κB in gene induction could regulate the acetylation of PARP-1 is currently under investigation.

Taken together, these results support the hypothesis that the different physiological functions of PARP-1 are regulated by post-translational modifications such as acetylation in a stimuli-specific manner.

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6 Unpublished results

6.1 MYBBP1a cellular localisation

6.1.1 Introduction and Aim

Section 5.2 shows that MYB binding protein 1a (MYBBP1a) represses NF-κB dependent transcription despite the fact that myc-MYBBP1a localises mainly to the nucleoli in non-stimulated Jurkat cells. The nucleolar localisation of MYBBP1a raises the question as to how MYBBP1a can repress nucleoplasmic RelA(p65) dependent transcription. It is possible that the small amount of MYBBP1a seen diffusely in the nucleus is the pool of MYBBP1a responsible for the observed repression. It is also possible that MYBBP1a could function by sequestration of RelA(p65) in the nucleolus. Repression of RelA(p65) by nucleolar sequestration has been shown previously [234]. Therefore, the localisation of MYBBP1a in the nucleolus may be important for its function. In 293T cells, flag tagged MYBBP1a was shown to be able to shuttle from the nuclear/nucleolus to the cytoplasm [235]. The aim of this work was to determine whether NF-κB activating signals could regulate the localisation of MYBBP1a in Jurkat cells.

6.1.2 Result

In order to investigate whether NF-κB activating signals could regulate the localisation of MYBBP1a immunoflorescence was performed in Jurkat T cells. Due to the lack of MYBBP1a specific antibodies appropriate for detection of endogenous MYBBP1a by immunoflourescence, myc tagged MYBBP1a was overexpressed in cells and detected with an anti-myc antibody as described in Section 5.2. In non-treated cells, MYBBP1a was detected in the nucleolus (Figure 10). In cells treated with 17.5 nM PMA (the same concentration that was used in the transient transfection reporter experiments in Section 5.2) myc-MYBBP1a was localised in most transfected cells (85 %) in the nucleoli, with just a few cells showing myc-MYBBP1a cytoplasmic staining.

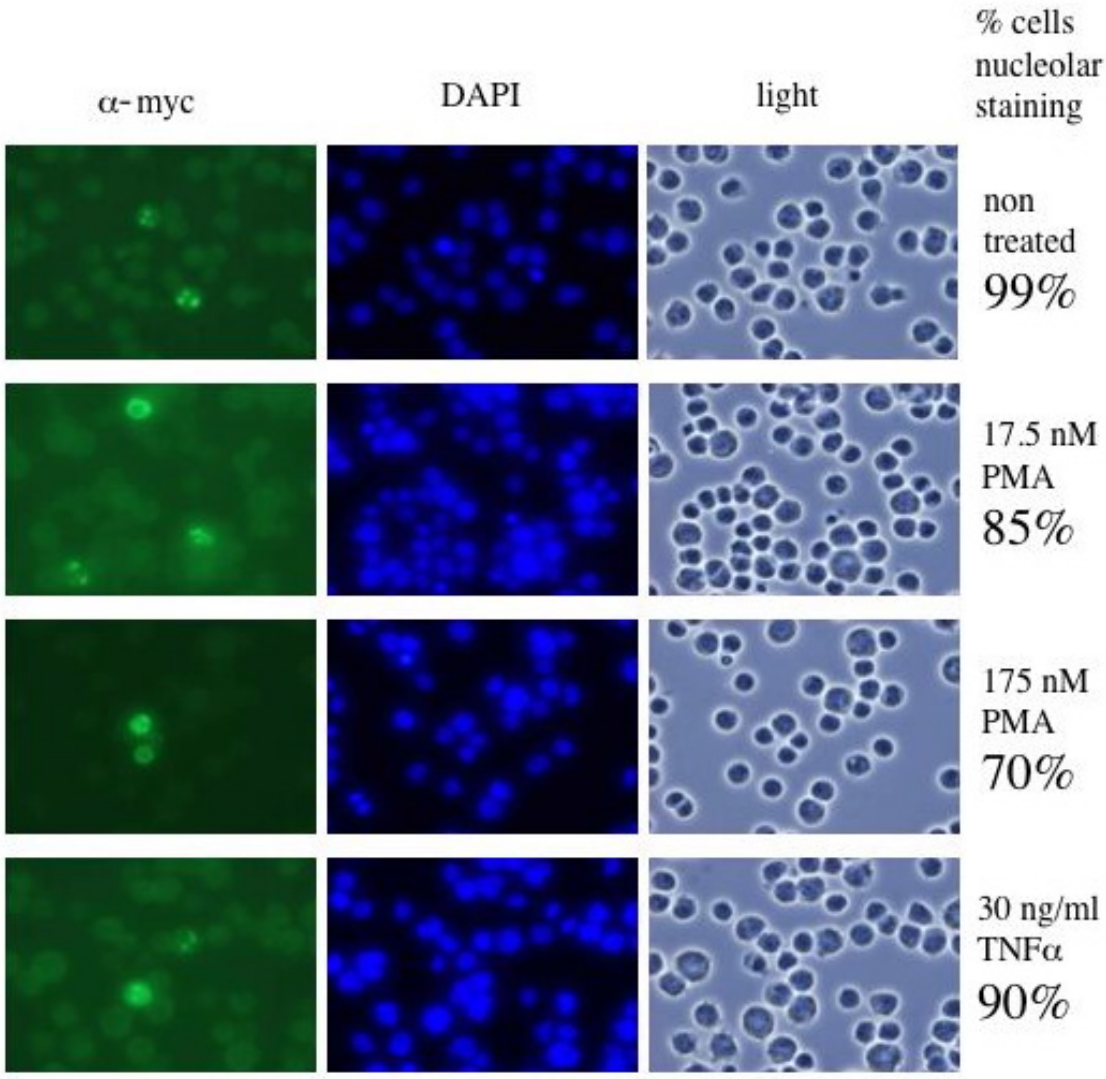


Figure 10. Detection of myc-MYBBP1a in Jurkat T cells by immunoflourescence. Myc tagged MYBBP1a was over-expressed in Jurkat T cells. The cells were stimulated for 1 hour with 17.5 nM PMA, 175 nM PMA or 30 ng/ml TNFα as indicated. Over 100 transfected cells were scored for nucleolar localisation, and the results are given as the % of transfected cells showing nucleolar staining. Bright green signals are from transfected cells, whilst the faint green signal is background. The experiment was repeated twice for non-stimulated and 175 nM PMA stimulated samples with similar results.

When the cells were stimulated with 175 nM PMA, approximately 70 % of transfected cells showed myc-MYBBP1a localisation mainly in the nucleolus. The rest of the transfected cells showed myc-MYBBP1a mainly in the cytoplasm and some in the nucleus. The effect of another NF-κB stimuli, TNFα, on the sub-cellular localisation of myc-MYBBP1a was also tested. Upon 1 hour TNFα treatment, 90 % of the transfected Jurkat cells showed mainly nucleolar staining for myc-MYBBP1a. These results indicate that MYBBP1a is found mainly in the nucleolus of Jurkat cells,

but can also be found in other sub-cellular compartments, upon cellular stimulation. Stimulation of Jurkat cells with PMA, a protein kinase C activator, resulted in a small pool of MYBBP1a being relocated to the cytoplasm.

6.2 PIKK family involvement in NF-κB dependent transcription

6.2.1 Introduction and Aim

We found that among other proteins, TRRAP (transactivating/transformation domain associated protein), ATR (ATM and rad3 related) and DNA-PKcs (DNA dependent protein kinase-catalytic subunit) could bind to the TAD of RelA(p65) (Section 5.1). These are homologous proteins of the phosphoinositide-3-kinase-related kinases (PIKK) superfamily. Another member of this family is ATM (Ataxia Telangiectasia). ATM, ATR and DNA-PKcs are serine/threonine kinases that are activated upon DNA damage to phosphorylate proteins involved in cell cycle progression, DNA replication, DNA repair and cell death (reviewed in [236], [237]). ATM and ATR can bind DNA directly and DNA-PKcs associates with DNA via ku80 and ku70. Upon DNA damage, they form foci in the nucleus together with other DNA damage response and effector proteins. One of these foci proteins is BRCA1, an NF-κB interacting protein.

ATM and ATR have distinct but overlapping roles. ATM is activated upon DNA damage caused by irradiation and other factors and is autophosphorylated within minutes (reviewed in [236], [237]). It is thought that it senses damaged DNA through recognition of aberrant chromatin structure. ATR is typically activated upon DNA damage caused by UV and stalled replication forks. DNA-PK is activated by DNA damage and is also involved in non-homologous end joining. Since DNA damage signalling pathways often lead to a change in gene expression, it is not surprising that these proteins have also been shown to have a role in transcription regulation. ATM binds to HDAC1 and ATR to HDAC2 and the DNA damage induced foci contain TFs like E2F1. All three kinases, especially DNA-PK, have been shown to phosphorylate p53, and activate p53 dependent apoptosis genes (reviewed in [238]).

The first indication that these proteins may be involved in the NF-κB response came from the observation that ionizing γ-irradiation (IR) led to activation of DNA

binding by NF-κB [239]. ATM was shown to be important for this pathway as cells lacking active ATM (AT5BIVA) showed no IR induced IκBα degradation, NF-κB DNA binding or activation of HIV reporter expression. Overexpression of ATM in these cells could rescue IκBα degradation, and IκBα was shown to be phosphorylated by ATM *in vitro* [240]. Also, protein extracts from ATM-/- mice were deficient in IR induced IKK activity and NF-κB DNA binding activity [241]. In the same study, protein extracts from DNA-PK -/- mice were not deficient in IR induced NF-κB binding activity. However in a different cellular system, DNA-PK was found to be involved in activation of NF-κB. In cells lacking active DNA-PK (M059J) IR irradiation did not lead to NF-κB DNA binding activity, and DNA-PK could phosphorylate IκBα and IκBβ *in vitro* [242]. The discrepancies may be due to the use of different cellular systems, since it is known that deficiencies in one PIKK family member can also affect other family members, and that the cells are chromosomally unstable and accumulate many mutations upon passaging.

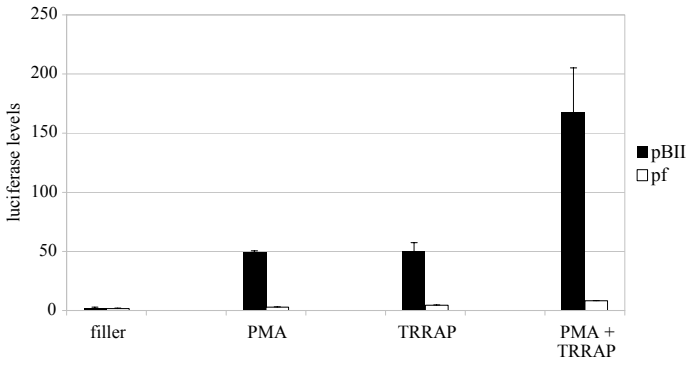
The function of TRRAP is slightly different since it does not contain kinase activity. Instead it is a member of many HAT complexes, including SAGA, GCN5, P/CAF, Tip60, TFTC and STAGA complexes (reviewed in [243]). TRRAP and its yeast homologue (Tra1) interact with many TFs, bridging DNA binding proteins to HAT complexes at various promoters. TRRAP disruption in mice leads to embryonic lethality [244] and many genes were found to be down-regulated in conditional knock out cells [245].

The aim of this work was to perform preliminary experiments to investigate the hypothesis that the direct interaction of a PIKK family member with the RelA(p65) TAD could lead to regulation of the transcriptional potential of NF-κB, through phosphorylation of RelA(p65).

6.2.2 TRRAP may activate NF-κB dependent transcription

Transient transfection reporter assays were carried out with reporter constructs in 293T cells. The reporter constructs were based on the pGL2 vector with the luciferase under the control of the minimal fos promoter (pf) or the same construct with two consensus NF-κB sites inserted (pBII). As expected PMA stimulation led to an increase in luciferase reporter gene expression of pBII (Figure 11A).

A.



B.

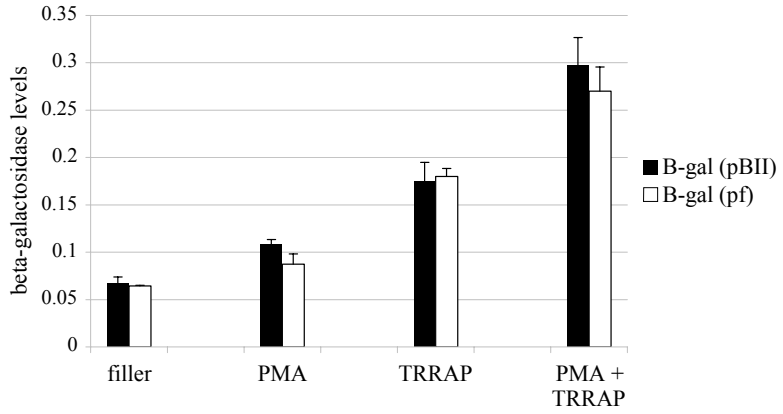


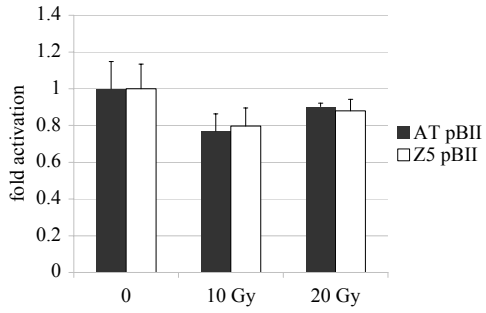
Figure 11. Functional assay showing the effect of TRRAP over-expression on transcription. **A**. TRRAP activates transcription of the NF-κB reporter, pBII. The numbers are relative luciferase levels $*10^3$. **B**. TRRAP also slightly activates transcription from another NF-κB independent promoter (RSV-βgal). Each condition was in duplicate, the bars indicate the mean and the error bars indicate the standard deviation. The experiment was performed twice, with TRRAP activating pBII driven reporter expression both times, but to very different extents. The experiments need to be repeated, confirmed and extended to be confident that TRRAP is acting as a cofactor of NF-κB regulated transcription.

TRRAP activated reporter gene expression to the same extent as PMA treatment. However, TRRAP also slightly increased the levels of the pf reporter and the β-galactose expression controlled by the constitutive RSV promoter (internal control). This indicates that TRRAP might increase general transcription in this cellular system. PMA treatment and TRRAP over-expression further increased pBII reporter expression. Again, these conditions also activated pf driven luciferase and RSV driven β-galactose expression, although to a lesser extent than the pBII driven luciferase expression.

6.2.3 ATM may be to be required for UV activation of NF-κB

To test if ATM could influence the transcriptional potential of NF-κB transient transfection reporter assays were carried out in ATM mutant cells (AT) and the same cells complemented with flag tagged ATM (Z5). Cells were stimulated with ionizing radiation (IR) at levels that have been shown to activate NF-κB or with other NF-κB activating signals (TNFα, etoposide, camptothecin (CPT) or UV). IR had no effect on reporter expression at 10 Gy (gray) or 20 Gy in AT (no functional ATM) or Z5 (ATM functional) cells (Figure 12A). This is unexpected since IR was shown to activate NF-κB DNA binding activity as well as HIV reporter expression in ATM functional cells [239, 240]. TNFα stimulated pBII reporter gene expression in Z5 (ATM functional) and AT (no functional ATM) cells, however the activation was higher in AT cells (Figure 12B). UV did not stimulate reporter expression in AT (no functional ATM) cells, but did stimulate NF-κB dependent reporter expression in Z5 (ATM functional) cells (Figure 12B). This indicates that functional ATM was required for UV stimulation of NF-κB dependent transcription in this experiment.

A.



B.

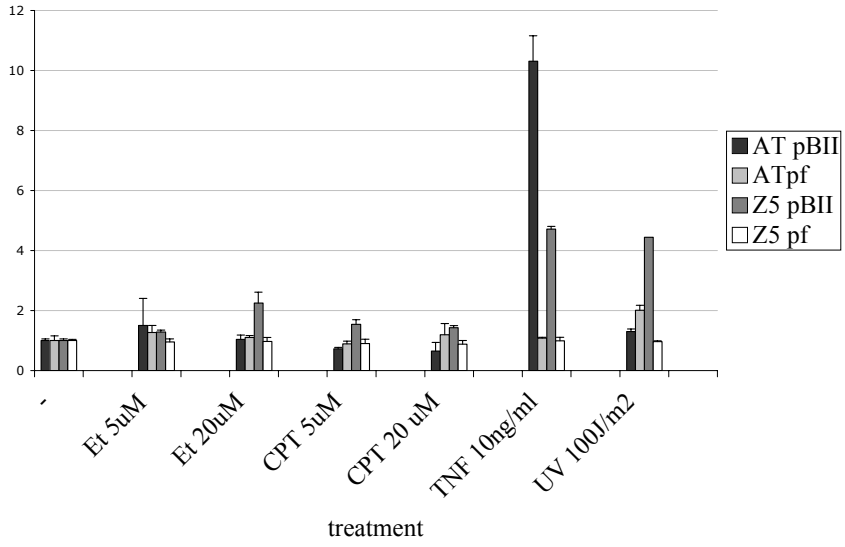


Figure 12. Functional assay to show the role of ATM in NF-κB dependent reporter expression. A. Cells containing functional ATM (Z5) or cells deficient for ATM (AT) were transfected with the NF-κB reporter gene pBII and stimulated with IR as indicated. **B**. AT or Z5 cells were transfected with pBII (contains NF-κB sites) or pf (no NF-κB sites) and stimulated with Etoposide (Et), camptothecin (CPT), TNFα or UV as indicated. Each condition was in duplicate, fold activation is the mean of the luciferase level/β-galactose level, where the filler sample (-) is set as 1. The error bars indicate the standard deviation. The experiment was performed only once, and needs to be confirmed.

6.2.4 RelA(p65) is phosphorylated by a DNA associated kinase

To test if a DNA associated kinase could phosphorylate RelA(p65) *in vitro* kinase reactions were performed. DNA associating kinases from HeLa extracts were immobilised on DNA cellulose and used to phosphorylate RelA(p65) or GST-p53 as a positive control or GST as a negative control (Figure 13).

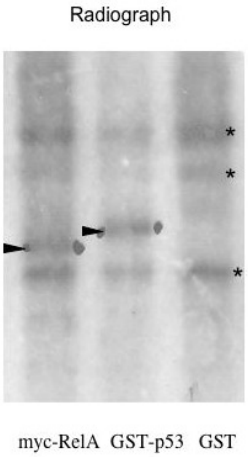


Figure 13. In vitro kinase assay. DNA associated kinases from HeLa cell extract were used to phosphorylate myc tagged RelA(p65), GST-p53 or GST in the presence of ^{32}P-γATP. The phosphorylated proteins were separated by SDS-PAGE and exposed to film. The experiment was repeated many times, with similar results. Arrow heads indicate the position of phosphorylated myc-RelA(p65) or GST-p53. Asterix indicates non specific phosphorylation of nuclear extract proteins.

As expected GST-p53 was phosphorylated while GST was not. His-myc-RelA(p65) was found to be phosphorylated by a DNA associated kinase. The DNA associated kinase could be a DNA binding kinase or a kinase associated with a DNA binding complex.

6.3 Materials and Methods

6.3.1 Functional assays

For the ATM transient transfection reporter assays the AT cells used are AT22IJE-T fibroblasts which are SV40 transformed cells from a skin biopsy of a female A-T patient [246]. Z5 cells are these cells stably transfected with flag-ATM protein. AT or Z5 cells were transfected with 2 μg pBII-luc or pf-luc reporters plus 2μg RSV-β galactose internal control using the calcium phosphate method. 6 h after transfection the medium was changed. Cells were stimulated with 10 or 20 Gy of gamma irradiation, 5 or 20 μM etoposide (inhibits topoisomerase II), 5 or 20 μM camptothecin (inhibits topoisomerase I), 10 ng/ml TNFα, or 100 J/m^2 UV and harvested approximately 6 h later.

For the TRRAP transient transfections, experiments were performed as above, but 293T cells were transfected with 0.5 μg pBII or pf reporter, 0.2 μg RSV-β galactose, and 5 μg TRRAP expression vector (flag tagged TRRAP in bluescript

based vector under the control of a CMV promoter provided by Prof. Michael Cole) or 2.5 μg of empty vector filler. The cells were treated with 175 μM PMA for 4 h. All conditions were performed in duplicate.

6.3.2 HeLa extract preparation

Approximately 100 million HeLa cells were harvested and washed in PBS + 0.2 mM PMSF. Cell pellets were resuspended in LBS (10 mM HEPES, 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, pH 7.2 + 0.1 mM DTT + 0.2 mM PMSF), washed in LBS, centrifuged and then resuspended in 2.5 cell volumes of LBS. The cell suspension was incubated on ice for 10 min and then flash frozen. The cell suspension was thawed quickly, protease inhibitors were added and the salt was adjusted to 0.5 M NaCl, 10 mM $MgCl_2$, 5 mM DTT. The cell suspension was incubated on ice for 3 min and centrifuged at 10 krpm, 3 min at 4 °C. The supernatant was saved and the pellet re-extracted. The supernatent was flash frozen and centrifuged at 14 krpm, 10 min at 4 °C before use.

6.3.3 GST and GST-p53 purification

GST and GST-p53 expression plasmids were transformed into *E. coli* strain BL21. Expression was induced for 4 h with 0.5 mM IPTG when bacteria were at O.D. 0.6. Bacteria were harvested and lysed by french press in 50 mM tris pH 7.5, 120 mM NaCl, 5 mM DTT, 0.5 % NP40, 1 mM PMSF. Lysates were incubated with Glutathione sepharose beads (Amersham) (500 μl beads for 1L culture in 15 ml lysis buffer) at 4 °C for 1 h. Bound material was washed 3 X PBS, 1 X lysis buffer + 200 mM NaCl, 1 X PBS. Protein was eluted 2 X elution buffer (50 mM tris pH 8, 10 mM reduced glutathione, 50 mM NaCl, 50 mM NaF, 2 mM $NaVO_4$, 50 mM β-glycerophosphate, 0.5 mM PMSF).

6.3.4 His-myc-p65 purification

His-myc-p65 was expressed in insect *Sf*21 cells using a baculovirus expression system. Three days after infection, the insect cells were harvested, washed in PBS, and lysed by homogenising in 10 mM tris pH 7.5, 500 mM NaCl, 10 % glycerol, 0.1 % NP40, 15 mM imidazole, 2 mM β-mercaptoethanol. The protein was purified in batch using nickel sepharose (Amersham), with elutions in 10 mM tris pH 7.5, 200 mM NaCl, 10% glycerol, 0.2 % NP40, 250 mM imidazole, 2 mM β-mercaptoethanol,

0.2 mM PMSF.

6.3.5 Kinase reaction

DNA cellulose (Sigma) was washed, then incubated 5-10 min with 1V HeLa extracts to 4V dilution buffer (50 mM HEPES, pH 7.5, 0.5 mM DTT, 10 mM $MgCl_2$ + protease inhibitors). Bound material was centrifuged at 1000 rpm for 10 seconds. The pellet was washed three times with wash buffer (25 mM HEPES pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 10 % glycerol) and one time with kinase buffer (wash buffer + 10 mM β-glycerophosphate, 10 mM NaF, 2 mM $NaVO_4$). The substrates were aliquoted into fresh tubes, 20 µl of DNA cellulose/kinase was added to each tube, followed by 1 µl 2.5 mM ATP and 1 µl 10 µCi/ul ^{32}P-γATP. The reaction was performed at 30°C for 10 minutes. To stop the reaction SDS containing loading buffer was added, and the reactions were analysed by SDS-PAGE and autoradiography.

Discussion

7 Summary of Results

The aim of this work was to identify cell type specific cofactors of RelA(p65) dependent transcription. The first step was to isolate proteins that could interact with the TAD of RelA(p65) from different cell types. This was achieved by performing GST pull down experiments using GST-RelA(p65)-TAD as bait and nuclear extracts from three different cell types as prey. Liquid chromatography-mass spectrometry was used to identify the proteins that bound to the GST-RelA(p65)-TAD. Many novel TAD interaction proteins were found, and some were found at higher levels when isolated from the nuclear extracts of certain cell types. For example, MYB binding protein 1a (MYBBP1a) was found to bind RelA(p65)-TAD at higher levels from Jurkat T cell nuclear extracts, than from HeLa or THP1 nuclear extracts. The function of MYBBP1a was investigated further, since it was previously shown to regulate the activity of other TFs. The interaction between MYBBP1a and RelA(p65) was confirmed with purified proteins and MYBBP1a was found to repress NF-κB dependent transcription. MYBBP1a could compete with the NF-κB co-activator p300 for TAD binding. It inhibited transcription activation at a step before pre-initiation complex formation. These results indicate that MYBBP1a is a co-repressor of NF-κB dependent transcription and is enriched in Jurkat T cells compared to HeLa epithelial cells or THP1 monocytes.

8 Interaction partners of RelA(p65)-TAD

8.1 Interaction Proteomics

In order to identify cofactors of RelA(p65) dependent transcription, the proteomic approach used was to isolate RelA(p65)-TAD binding proteins from nuclear extracts of different cell types. A high amount of GST-RelA(p65)-TAD (bait) and nuclear extract (prey) was used with stringent binding and wash buffer conditions. An alternative procedure would have been to isolate proteins that bind to full length RelA(p65). This was not attempted since it is known that most cofactors of transcription interact with the TAD of RelA(p65), as this is the domain responsible for most of the transactivating potential of RelA(p65) [110]. Also, there is evidence that full length RelA(p65) forms a closed structure when not phosphorylated at serine 276 and this closed structure is incompatible with interaction with the cofactor p300 [99]. Another alternative would have been to perform immunoprecipitation of tagged RelA(p65) and analyse the co-immunoprecipitated proteins. The advantage of this would be that the lower amount of bait used would isolate less false positive interactions. However, a previous report using TAP-tagged RelA(p65) failed to identify any known cofactors of transcription [247]. By using a relatively high amount of the specific functional domain, we were able to isolate known protein interaction partners of RelA(p65)-TAD.

Nuclear extract was used as the prey since most cofactors of transcription are present in the nucleus. Any cofactors present in the cytoplasm of unstimulated cells would not be identified with this approach. Whole cell extracts were not used as nuclear proteins would be diluted with cytoplasmic proteins in the whole cell extract leading to a reduced probability of forming a favourable cofactor interaction with the prey. The nuclear extracts were made from large cultures (2L) of Jurkat T, THP1 monocyte, or HeLa suspension cells. All the cells types were chosen as they contain inducible NF-κB activity and they are easy to culture. It is very time consuming and expensive to culture the required amount of cells from tissues or primary cell, so transformed cell lines were used. However, the protein constituents and metabolism of these cell lines may not be representative of their primary cell counterpart.

As shown in Table 2, Section 2.5.1, there are many different signalling events leading to the phosphorylation of specific sites in the TAD of RelA(p65). However, non-modified TAD was used in the proteomic approach, therefore proteins with the ability to interact only with specifically phosphorylated TAD could not be isolated. It would be very informative to repeat the GST-pull down experiments using mutant versions of TAD. A glutamic acid residue partially resembles the structure of a phosphorylated serine, and can be used to mimic a specific phosphorylatable serine. A mutant form of RelA(p65)-TAD could be used to isolate interaction proteins that have a high affinity for the specifically phosphorylated TAD.

Whilst interpreting the results, it is important to realise that all interaction partners that were isolated are proteins that have the ability to interact directly or indirectly with TAD in this system, but may not interact physically or functionally with RelA(p65) *in vivo*. For example, many chaperones were found to interact from all cell types with GST-RelA(p65)-TAD, but are unlikely to do so in the cell to regulate NF-κB transcriptional function. However, many of the novel TAD interaction proteins found may have a role in RelA(p65) dependent transcription. To investigate this further it would be necessary to confirm the specificity of the interactions and to perform functional assays.

8.2 Protein Separation and Identification

The protein interactors of RelA(p65)-TAD were separated by gel electrophoresis and the high molecular weight proteins (above 100 kDa) were identified by mass spectrometry. Two approaches were used to separate the RelA(p65)-TAD interacting proteins. The first approach was separation of individual proteins by gradient 1D SDS-PAGE. This approach allowed separation of the dominant GST-RelA(p65)-TAD band from the proteins of interest. Another approach would have been to use 2D gel electrophoresis. This is normally the separation of proteins according to isoelectric point (the pH at which a protein has no charge), and then according to mass. The advantage of 2D gel electrophoresis is that it separates the proteins more thoroughly into individual spots. This would have helped to separate band 3 and 4 containing p300, CBP and Down Regulated in Metastasis (DRIM) in the Figure 1 of Section 5.1, which would have enabled comparisons of these proteins between the cell types. The disadvantage of 2D gel electrophoresis is that specialised equipment is required and the procedure is longer. We used Sypro

Ruby (Molecular Probes) to visualise protein bands on the 1D SDS-PAGE. Sypro Ruby is thought to be as sensitive as the conventional method of silver staining [248]. Advantages of using this staining technique is that it interacts with proteins non-covalently and relatively independently of the amino acid sequence. Therefore, Sypro Ruby staining does not interfere with mass spectrometry analysis and can be used to quantify stained proteins. Silver staining involves silver covalently interacting with cysteine residues which can interfere with mass spectrometry analysis. The strength of the silver staining of each protein is dependent on the number of cysteines within the protein, so silver staining is less quantitive.

The second approach we used to separate the proteins was a "shot gun" approach. A short (2 cm) minigel was used to separate the GST-RelA(p65)-TAD from the proteins of interest. The gel piece containing a large mixture of proteins of interest was then cut into smaller pieces before trypsin digestion. The advantage of this approach is that all proteins were present in the gel, so no faint bands could be missed. With this approach, many Mediator subunits were identified that were not detected in the first approach.

After tryptic digestion, liquid chromatography-tandem electrospray mass spectrometry was used to identify the proteins isolated by both separation approaches (reviewed in [249]). The instrument used was adapted for high throughput accurate analysis of complex protein/peptide mixtures. High performance liquid chromatography separates peptides according to how hydrophobic they are by use of a reverse phase (hydrophobic) column. The mass/charge ratio of all the peptides was then determined by mass spectrometry, which consists of ionisation, mass analysis and detection. Ionisation of the sample was performed by electrospray ionisation. In this method of ionisation, the peptide mixtures dissolved in a solvent are forced through a charged metal capillary. Droplets are then formed, normally containing positively charged peptide ions. The repulsion of the positive charges forces the droplets into the gas phase, and evaporation and collision between droplets separates each charged peptide. The major advantage of using of electrospray ionisation rather than the other common method of ionisation, matrix-assisted laser desorption/ionization, is that electrospray is easily coupled to liquid chromatography. After ionisation, the peptides are directed to the mass analyser, in our case a quadrupole-time of flight. The mass analyser guides the charged peptides to the detector, based on their mass/charge ratio. We used tandem mass spectrometry, a

method to perform multiple rounds of mass spectrometry. Each round involved separation of peptides and dissociation of each peptide into smaller pieces by collision induced dissociation. The data collected was analysed with the program MASCOT, and peptide sequences could be calculated from the mass/charge ratio of the many peptide fragments formed. Overall, the "shot gun" approach allowed identification of proteins below the detection limit of Sypro Ruby staining (about 1 ng/band), from a large protein mixture.

Using both approaches many different proteins were identified as RelA(p65)-TAD interacting partners [250]. The known RelA(p65)-TAD interacting co-activators, p300 and CBP were identified, indicating that the approach used was suitable for the identification of high molecular weight novel transcription cofactors of RelA(p65). Many hypothetical proteins were also found to interact with the TAD of RelA(p65), indicating that they may be involved in transcriptional regulation by RelA(p65). Other proteins with known cellular functions were identified and are discussed in the next sections. Some proteins were found at higher levels to bind the RelA(p65)-TAD from specific cell types. The cell type enriched binding could be due to higher expression levels of the protein in particular cell types, or due to cell type specific PTM of proteins regulating the interaction with RelA(p65)-TAD.

8.3 New RelA(p65)-TAD interaction partners

8.3.1 TRRAP

Many proteins were discovered to interact with the TAD of RelA(p65) that were previously not known to do so, including the transcription regulator TRRAP (transactivation/transformation-domain associated protein) (Section 5.1). Preliminary experiments indicate that TRRAP may be a co-activator of NF-κB dependent transcription (Section 6.2.2). TRRAP is a member of many HAT complexes known to be involved in transcription activation (reviewed in [243]). Yeast have a homolog of TRRAP called Tra1. The CT of Tra1 was shown to bind to other acidic transcription activation domains (VP16 and GCN4), and Tra1 recruited HAT activity to promoter regions [251]. TRRAP has also been shown to be recruited to promoters by mammalian TFs, like Myc [252]. It has been shown that TAFs [117] and P/CAF [173] activate NF-κB dependent transcription, and these proteins are often present in TRRAP containing complexes. Therefore, the mechanism of NF-κB activation by

TRRAP could also be through the recruitment of HAT complexes to the RelA(p65)-TAD. Recruitment of TRRAP containing HAT complexes to RelA(p65)-TAD could result in the acetylation of the surroundings, including histones and non-histone proteins at promoters. In this hypothesis, TRRAP would directly interact with the TAD of RelA(p65), which has yet to be investigated.

Many TRRAP complexes also contain TAFs. TAFs that are known to interact with RelA(p65)-TAD are TAF1 ($TAF_{II}250$), TAF6 ($TAF_{II}80$) and TAF11 ($TAF_{II}28$) [117]. Both TRRAP and TAF6 are present in SAGA [253] and TFTC [254] and it is likely that other complexes also contain TRRAP and RelA(p65)-TAD interacting TAF subunits. Unexpectedly, the high molecular weight TAF1 was not found in our search for RelA(p65)-TAD interacting proteins. This could be due to its presence at concentrations too low for detection, or because it does not interact at the stringent conditions that we tested.

There is also a possibility that TRRAP containing P/CAF complexes could acetylate RelA(p65) since recombinant P/CAF was shown to acetylate lysine 122 and 123 in the RHD of RelA(p65) [169]. Acetylation at these sites led to termination of NF-κB dependent transcription, implying that if TRRAP containing P/CAF complexes acetylate these sites *in vivo*, TRRAP would be functioning as a terminator of NF-κB dependent transcription. On a simple level, this would not be in agreement with the hypothesis that TRRAP is a co-activator of transcription. However, transcriptional regulation is complex and it is possible that a transcriptional activator can also be a transcriptional terminator. This is the case with p300, which is recruited to estrogen receptor response genes within 1 hour of estrogen stimulation to activate transcription [255], however, upon continued stimulation, p300 mediated the acetylation of the transcription co-activator SRC-3 (ACTR). This acetylation event reduced the interaction between SRC-3 and the estrogen receptor, and led to attenuation of transcription.

The importance of TRRAP in normal cellular activity is apparent from the lethal phenotype of TRRAP-/- cells [244]. TRRAP was found to be essential for cell proliferation and mitotic exit. In a conditional knock out cell line, TRRAP was found to regulate transcription of a wide variety of genes [245]. Loss of TRRAP led to down regulation of approximately 70% of the TRRAP regulated genes, indicating that TRRAP acts mainly positively on transcription (but also negatively). The failure of

TRRAP deficient cells to proliferate was found to be due to defective transcription of the mitotic checkpoint proteins, Mad1 and Mad2 [256]. It would be useful to know which NF-κB dependent genes are regulated by TRRAP, and whether they are also genes important for controlled proliferation.

8.3.2 DNA-PK, ATR and ATM

Ionising radiation has been shown to activate NF-κB, and this has been suggested to be dependent on DNA-PK or ATM mediated phosphorylation of IκB [241, 242]. Here, ATR and DNA-PKcs were found to interact with RelA(p65)-TAD (Section 5.1), and a DNA associated kinase could also phosphorylate RelA(p65) (Section 6.2.4). Also, preliminary experiments indicated that ATM might be important for a UV mediated NF-κB response (Section 6.2.3). These results support a hypothesis that genotoxic stress could activate NF-κB through the IκB degradation pathway, as well as interacting with the TAD to regulate NF-κB transcriptional potential, possibly by phosphorylation of specific residues. This would be similar to the mechanism of p53 activation in response to genotoxic stress. Upon ionising radiation and activation of ATM, the inhibitors of p53 (MDM and MDMX) are phosphorylated and inactivated [257, 258]. p53 is also directly phosphorylated by ATM, at residues that enhance its transcriptional activity [259]. Experiments to further investigate the hypothesis that genotoxic stress could trigger ATM, ATR or DNA-PK mediated phosphorylation of RelA(p65) to enhance transcription of specific genes are required. Phosphorylation experiments using recombinant proteins could be used to identify which of the kinases (if any) have the ability to phosphorylate specific threonines or serines within RelA(p65). These experiments could be followed by *in vivo* phosphorylation experiments and functional assays. The use of siRNA knock down cells would be beneficial, as the genetic instability seen in ATM-/-, and DNA-PK-/- mouse cells would be largely avoided due to the shorter duration of the experiments.

DNA-PKcs has a role in non-homologous end joining (NHEJ) in the repair of DNA damage and also in V(D)J recombination [237]. It is possible that the interaction seen between DNA-PK and RelA(p65) is due to involvement of DNA-PK in NF-κB dependent transcription, or involvement of NF-κB in DNA-PK mediated NHEJ. Interestingly, there is a link between the two processes, since NF-κB is

involved in germline transcription of light chain genes, which is necessary for recombination [260].

8.3.3 Mediator Subunits

In the "shotgun" proteomic approach many Mediator subunits from nuclear extracts were found to interact with the TAD of RelA(p65) and *in vitro* translated Med14, Med24 and Med17 subunits bound weakly to RelA(p65)-TAD (Section 5.1). This confirms a previous study from Näär *et al*. [120] where the whole Mediator complex was found to interact from nuclear extracts with RelA(p65)-TAD. However, we could not detect Mediator subunits when analysing each band indicating that in our study the proteins were present at levels below the detection limit of Sypro Ruby staining. This is interesting since in the previous study, Mediator subunits were the main interaction partners of RelA(p65)-TAD as shown by silver staining [120]. It is likely that in our experiment Mediator interacts with a lower affinity than in the experiments performed by Näär *et al*. Both experiments were GST pull down experiments with HeLa nuclear extracts prepared in similar ways. The main difference in experimental conditions are that Näär *et al* used a wash buffer containing 250 mM KCl, whereas we used a similar buffer, but containing 400 mM KCl. Other small differences in procedures are that they used amino acids 434-551 of RelA(p65) (instead of 428-551), and approximately twice the amount of HeLa nuclear extracts. Therefore, it is likely that the Mediator/RelA(p65) interaction is sensitive to salt concentrations, due to a low affinity interaction or an indirect interaction (bridged by another protein).

The *in vitro* translated Mediator subunits Med14, Med24 and Med17 interacted very weakly with RelA(p65)-TAD (Section 5.1), indicating that either i) another Mediator subunit that we did not test or another protein forms the direct interaction with RelA(p65)-TAD, or ii) multiple Mediator subunits interact weakly with RelA(p65), to stabilise a higher order complex. Many RelA(p65) containing NF-κB dimers often bind to many κB sites within the same enhancer, and each TAD could interact with a subunit of the Mediator complex. Together with other TFs, which also interact with Mediator subunits, Mediator could be effectively recruited to the enhanceosome through multiple weak interactions.

Med14 and Med 17, two of the Mediator subunits that we found to interact with RelA(p65)-TAD also interact with other TFs (reviewed in [149]). Med14 (also called TRAP170/DRIP150) interacts ligand independently with the nuclear hormone

receptors glucocorticoid receptor ([261]) and hepatocyte nuclear factor 4 [262] as well as with signal transducer and activator of transcription 2 (STAT2) [263] and sterol regulatory element binding protein 1a (SREBP1a) [264] to activate transcription. Med17 (also called TRAP80/DRIP77) interacts with Dif (a *Drosophila melanogaster* RHD containing protein) [265], VP16 [266], p53 [266], heat shock factor [267] and STAT2 [263]. These observations indicate that STAT2 interacts with at least two of the same Med subunits as RelA(p65)-TAD. The transcription activation domain of STAT2 contains acidic residues (like RelA(p65)-TAD) [268]. However, alignment of STAT2 and RelA transcription activation domains shows no similarity between them.

Mediator has been shown to have different structural conformations depending on whether it is free, bound to RNAPII, or to different transcription factors [147, 269]. The conformation of the Mediator complex was similar when bound by TFs that interact with the same subunit of Mediator [270]. This indicates that each TF may interact with particular Mediator subunit(s) to form a specific conformation of Mediator. Each conformation of Mediator may have a distinct function. How conformation affects Mediator function remains to be determined. Conformations more or less able to form interactions with other TFs would result in the coordination and integration of different signals. Conformations more or less able to interact with GTFs and RNAPII would regulate the transcriptional outcome. This would lead to the precise regulation of transcription at specific enhanceosomes. If the conformation of Mediator were dependent on the Med subunit contacted by the TF, it would be expected that the Mediator bound to RelA(p65) would have a different conformation depending on which Med subunit was bound by the TAD. If RelA(p65)-TAD interacted with Med17 (as VP16) it may have a structure similar to the one seen when Mediator was bound to VP16 (Figure 14). If RelA(p65) bound to Med14 (as SREBP1a) it may have a structure similar to the Mediator bound to SREBP1a (Figure 14). The fact that RelA(p65) (and other TFs) can interact with more than one Mediator subunit indicates that the accumulation of TF/Mediator interactions might lead to a specific Mediator conformation and function.

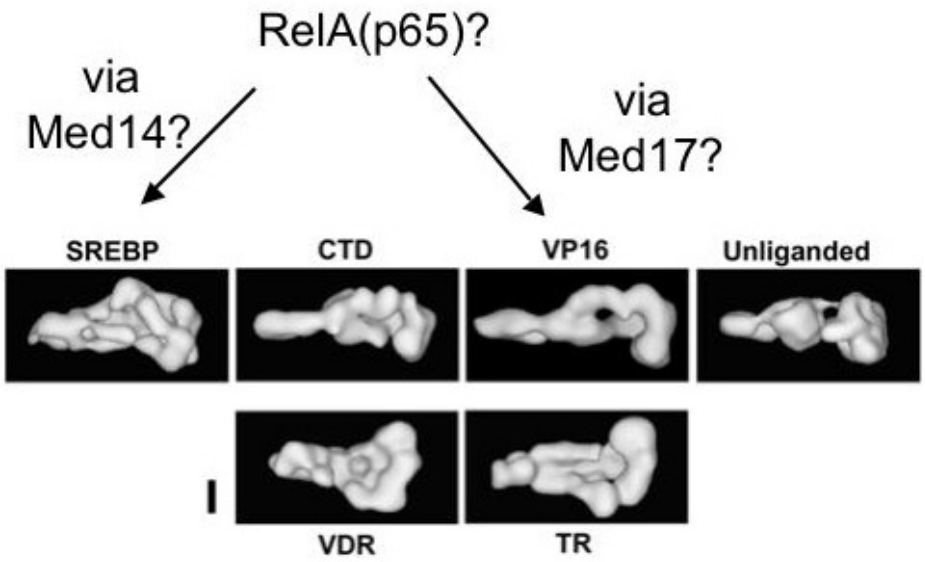


Figure 14. Electron micrograph of different conformations of the Mediator complex. Mediator forms several different conformations upon interaction with SREBP, RNAPII CTD, VP16, VDR or TR, or when alone (unliganded). A RelA(p65) bound Mediator complex might form a similar conformation, depending on whether it binds through the Med14 or Med17 subunit. The bar indicates 7.5 nM. Picture adapted from [270].

8.3.4 IQGAP1/3

IQGAP1 and IQGAP3 are homologous proteins that were also detected to be novel interacting partners of RelA(p65)-TAD. IQGAP1 promotes cell/cell adhesion and mobility by activation of GTPases [271], indicating RelA may have a function in these processes. It is also possible that these IQGAP proteins have a role in transcription.

9 MYBBP1a

9.1 MYBBP1a in RNAPII dependent transcription

9.1.1 Interaction with transcription factors

MYBBP1a was found to interact with RelA(p65)-TAD from all three cell lines, although the levels of MYBBP1a isolated from Jurkat nuclear extracts were much higher than from THP1 or HeLa nuclear extracts. MYBBP1a (originally called p160), and its proteolytically NT cleavage product (p67) were first identified as factors that could bind to the leucine zipper negative regulatory region of the transcription factor Myb [272]. The interaction between p67 and Myb seemed stronger than the interaction between MYBBP1a and Myb. Since its identification, MYBBP1a has also been shown to interact with other TFs. MYBBP1a, but not p67, was found to interact with the leucine zipper of c-Jun [272] and MYBBP1a could also interact with the acidic activation domain of the aromatic hydrocarbon receptor (AhR) [273]. However, it is not known whether these interactions between MYBBP1a and Myb, AhR or c-Jun are direct or via a bridging factor. MYBBP1a was found to interact with the negative regulatory domain of the peroxisome proliferator activated receptor γ (PPARγ) co-activator 1α (PGC-1α), probably through two leucine rich domains in PGC-1α [274]. PGC-1α could interact with the *in vitro* translated NT of MYBBP1a (p67) as well as the CT of MYBBP1a, indicating a probable direct interaction with at least two parts of MYBBP1a. In this thesis, an interaction between the acidic activation domain of RelA(p65) and MYBBP1a is shown, and is likely to be a direct interaction since there are only a few contaminants present (Section 5.2). It may be that MYBBP1a contains multiple protein interaction domains, for binding particular types of domain (Figure 15). The sequence of MYBBP1a contains two putative leucine zipper motifs, indicating that it may be able to dimerise with other leucine zippers. Also, many LXXLL (L = leucine X = any amino acid) motifs are present, which often form interaction surfaces with nuclear hormone transcription factors and other transcriptional regulators (reviewed in [275]). It would be interesting to map which region of MYBBP1a interacts with the acidic TAD of RelA(p65).

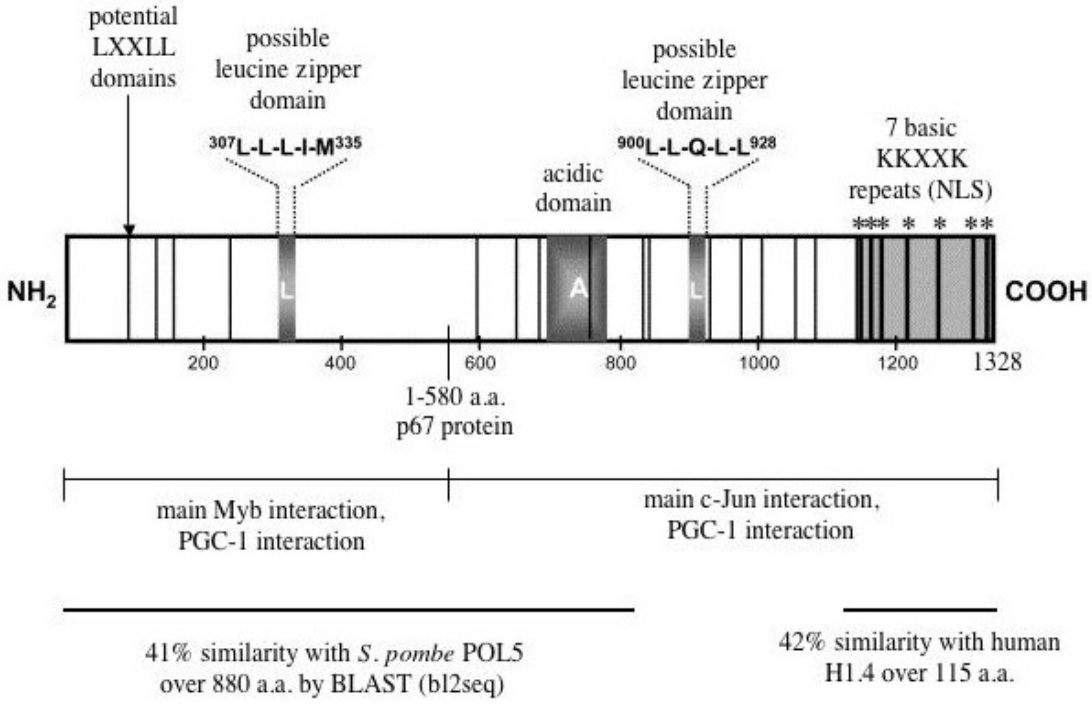


Figure 15. Features of MYBBP1a. Potential LXXLL motifs are indicated by black lines. The two possible leucine zipper domains are shown as shaded bars with a light L inside. The acidic domain is shown as a shaded bar with A inside. The CT was found to contain 7 nuclear localisation signal (NLS) motifs indicated with a *. Regions found to interact with Myb, c-Jun or PGC-1α are indicated. Sequence similarity with POL5 and H1.4 is indicated, as determined by BLAST [276]. K, lysine; X, any amino acid; L, leucine; I, isoleucine; M, methionine; Q, glutamine. Picture adapted from [235].

p67 is the CT proteolytical cleavage product of MYBBP1a approximately corresponding to residues 1-580. It was detected in mouse bone marrow lymphoblast (FDC-P1) cell extracts, but not in mouse embryonic fibroblasts (NIH3T3) cell extracts [277]. Neither p67 nor the yeast homolog POL5 contain the nuclear localisation signals mapped to be in the CT of MYBBP1a. However, endogenous p67 was found by western blot in nuclear extracts not cytoplasmic extracts [277] and POL5 has been detected by immunoflourescence in the nucleolus. A CT deletion construct of MYBBP1a was cloned and called p67* since the precise cleavage site for p67 is not known. Interestingly, flag-p67* was found by western blot to be cytoplasmic in HEK 293 cells [235]. However, the presence of p67 has also been suggested to be due to extract handling, since *Tavner et al.* claim that little p67 protein was seen when the cells were lysed directly in SDS-LB [277]. The putative

p67 protein was not investigated in this thesis, however it may be that it also has a role in NF-κB regulated transcription.

9.1.2 MYBBP1a as a transcriptional repressor

Previous work indicated that MYBBP1a could repress the transcription factors Myb and PGC-1α [274, 277]. Although full length MYBBP1a had little effect on Myb reporter gene expression, the N terminal of MYBBP1a (p67*) repressed transactivation in CV-1 (kidney fibroblast) cells in a concentration dependent manner, largely dependent on the leucine zipper of Myb [277]. Transfection of both MYBBP1a and p67* repressed Gal4-DBD-PGC-1α reporter expression in HIB1B brown fat cells, and this was dependent on the negative regulatory region of PGC-1α. MYBBP1a and p67* also repressed transcription of PPARγ and PGC-1α dependent reporter transcription. Adenoviral expression of MYBBP1a (but not p67*) in C2C12 myoblasts resulted in the repression of PGC-1α activation of mitochondrial respiration and cytochrome c and β-ATP synthase mRNA production. Gal4-DBD-MYBBP1a or Gal4-DBD-p67* could repress reporter transcription indicating MYBBP1a and p67* have intrinsic transcriptional repressive abilities. MYBBP1a also repressed NF-κB dependent reporter expression *in vivo* and *in vitro* (Section 5.2). However MYBBP1a does not generally repress all transcription factors, since expression of MYBBP1a increased AhR dependent reporter expression in mouse hepatoma cells [273] and had little effect on p53 dependent reporter gene expression (Section 5.2). This indicates that MYBBP1a might be a specific repressor of transcription for a select group of transcription factors.

9.1.3 Mechanism of MYBBP1a repression

There are multiple lines of evidence that MYBBP1a represses transcription as a classical transcriptional co-repressor. First, MYBBP1a was found to have intrinsic repressive ability since it repressed transcription when fused to the DBD of GAL4 in GAL4 dependent reporter assays [274]. Second, MYBBP1a repressed transcription without disturbance of PGC-1α binding to the promoter region of a PGC-1α activated endogenous gene (myoglobin) in C2C12 myotubes. Lastly, in this thesis I show that MYBBP1a could also repress transcription *in vitro*, on chromatin templates with RelA(p65) pre-bound (Section 5.2).

MYBBP1a may function as a co-repressor through multiple mechanisms, depending on the enhanceosome context. *In vitro,* MYBBP1a was found to repress NF-κB activated transcription at a step after TF binding to the chromatinised template, but before addition of GTF containing HeLa nuclear extracts (Section 5.2). This indicates that it represses transcription at a stage before PIC formation, which is the stage at which many transcriptional cofactors modify the nucleosomes in the promoter region. MYBBP1a was shown to compete with p300 for TAD binding, indicating that the repressive affect of MYBBP1a could be by displacement of the co-activator p300 (Section 5.2). This would lead to less acetylation of histones, or other non-histone proteins including RelA(p65) itself, which are thought to activate transcription. There is also some evidence that MYBBP1a mediated repression could be through recruitment of a HDAC containing co-repressor complex. GAL4-DBD-MYBBP1a repressed transcription was partially alleviated by treatment with the HDAC inhibitor TSA [274]. The HDAC proteins present in this possible HDAC/MYBBP1a complex remain to be identified. There is also evidence that protein stability of PGC-1α may be affected by MYBBP1a expression levels in some cell types [274]. MYBBP1a expression in brown fat HIB1B cells caused a decrease in the protein levels of expressed full length PGC-1α and 1-400 (containing the negative regulatory region that binds to MYBBP1a). However, MYBBP1a did not cause a decrease in the protein levels of a phosphorylation mimic of PGC-1α, which no longer has the ability to interact with MYBBP1a [274].

9.2 Cell type expression pattern of MYBBP1a

MYBBP1a mRNA is transcribed as a single 4.5 kb mRNA transcript to varying levels in all cell lines tested, including NIH 3T3 (embryonic fibroblast), J774 (macrophage), FDC-P1 (bone marrow lymphoblast), WEHI-3B (leukaemia) mouse cell lines [277] and HEK 293T (embryonic kidney epithelial cells), TF-1 (erythroleukemia), HeLa (cervical carcinoma epithelial), HFF2 (foreskin fibroblast) human cell lines [278] and in all mouse tissues tested [277]. Data from the publicly available Symatlas database [279] (http://symatlas.gnf.org/SymAtlas/) indicate that the amygdala (emotion processing part of brain), whole brain tissue, bone marrow CD105+ endothelial cells (similar to mesenchymal cells that differentiate into osteoblasts, chondrocytes, myocytes, adipocytes and neuronal cells), bone marrow CD34+ stem cells, 721 B

lymphoblasts (immature B lymphocyte), CD4+ (helper T cells) and CD8+ (cytotoxic T cells) have consistently high levels of MYBBP1a between different data sets and micro-array platforms. However, high levels of MYBBP1a protein cannot automatically be assumed to occur in these tissues, since proteolytical cleavage to p67 (if found not to be a handling phenomena) seems only to occur in some cell types. MYBBP1a protein was found in higher levels in Jurkat T cells, than in THP1 monocytes or HeLa epithelial cells, indicating that cell type specific differences in MYBBP1a protein levels do occur (Section 5.2). It is necessary to determine the MYBBP1a protein levels in many other cell types.

The expression pattern of MYBBP1a mRNA does not to completely overlap with the expression pattern of the transcription factors that are known to be regulated by MYBBP1a. RelA(p65) is constitutively expressed, and is found especially high in the cells of the immune system, including lymphoblasts and T cells [200]. PGC-1α is expressed in cells with high oxidative metabolism such as heart, skeletal muscle, kidney, brown fat, liver, and the brain (reviewed in [280]). Myb is expressed mainly in immature haematopoietic cells [281]. AhR expression is induced and is particularly high in the lung, liver, brain, heart, and spleen [282] while c-Jun expression is ubiquitous [283].

Control of transcription through cell type specific expression of cofactors has been suggested to be important for regulation of gene expression. Tissue restricted expression of a cofactor of transcription will regulate transcription of genes in those cells only. For example, the B cell specific BOB1/OCA-B co-activator is required for transcription of immunoglobulin genes in B cells by the ubiquitously expressed OCT1 (reviewed in [280]). Even greater selectivity can be achieved since cofactors are likely only to regulate selected promoters or enhancers. A cell type restricted cofactor could coordinate transcription of specific genes by various TFs. If the regulated genes are involved in the same cellular process, then coordinated regulation of their expression would lead to an extremely fast response during the regulation of the particular cellular process (reviewed in [280]). The cellular processes regulated by MYBBP1a are not known, but are likely to be important in the cell types where MYBBP1a levels are highest, possibly T, B and neuronal cells.

9.3 Function of MYBBP1a in the nucleolus

MYBBP1a is found mainly in the nucleoli of cells, the sub-nuclear organelles where rDNA transcription and ribosome synthesis occurs (reviewed in [284]). Within the nucleus there are 1-5 nucleoli, and together they contain a total of 250 copies of three of the four rDNA genes on 5 different chromosomes. MYBBP1a has a single homologue in animals, yeast and fungi [285]. The *S. cerevisiae* homologue, POL5, is also a nucleolar protein, with an essential function in the regulation of rRNA transcription [286]. It was found to bind near the enhancer region of rDNA repeats indicating it could be acting as a transcription cofactor for RNAPI. POL5 was first thought to be a new member of the B type polymerases by sequence homology of the conserved Pol domains, and recombinant POL5 was found to have polymerase activity *in vitro* [286]. However, POL5 purified from yeast had no detectable polymerase activity, and POL5 temperature sensitive mutants had no defect in DNA replication or DNA repair at the restrictive temperature. Therefore, POL5 does not have an essential function in DNA synthesis. Closer inspection of the POL5 amino acid sequence, showed that POL5, and therefore also MYBBP1a, is very unlikely to be a polymerase [285]. POL5 and MYBBP1a are predicted to be entirely α-helical, unlike polymerases, which are α-β fold proteins, where the catalytic site is within a β sheet. The Pol motifs are not present in MYBBP1a and only one full consensus Pol sequence was found to be conserved in POL5 [285]. Since POL5 is essential for viability, and rRNA synthesis, it is also possible that MYBBP1a has an important role in rDNA transcription regulation [286].

The nucleoli are also involved in other functions, such as cell cycle control, apoptosis, virus infection and cellular stress (reviewed in [287-289]). The nucleoli can regulate RNAPII transcription by sequestration of RNAPII transcription machinery [234, 290, 291]. Therefore the nucleolar MYBBP1a may have a function in these processes. RelA(p65) was found to be sequestered to the nucleolus upon cell treatment with the pro-apoptotic stimuli, aspirin, serum deprivation or UV-C [234]. Aspirin treatment led to IκBα degradation (after 2 hours), nuclear localisation of RelA(p65) and nucleolar accumulation (at 5-8 hours). Nucleolar sequestration of RelA(p65) coincided with a decrease in NF-κB reporter expression, and led to apoptosis after 16 hours of aspirin treatment [234]. Protein synthesis was required for the nucleolar localisation of RelA(p65). The nucleolar sequestration was dependent

on nucleolar localisation sequence in the NT of RelA(p65) . Transfection of a nucleolar localisation signal mutant of RelA(p65) led to a dominant effect, and no sequestration of endogenous wild type or transfected mutant RelA(p65) was observed [234]. There was also no transcription inhibition or apoptosis in these cells. An interesting possibility is that MYBBP1a could have a role in the nucleolar sequestration of RelA(p65) and other proteins. Further experiments to investigate this hypothesis would be to co-express RelA(p65) and MYBBP1a in cells, and determine the localisation of the proteins after treatments with various stimuli, such as aspirin.

10 RelA(p65)-TAD cofactors of transcription

10.1 TAD complex formation

In this work many proteins were identified to interact with the TAD of RelA(p65). These proteins, together with previously characterised TAD interacting proteins form a large number of potential transcriptional cofactor complexes capable of interacting with the TAD of RelA(p65) (Figure 16).

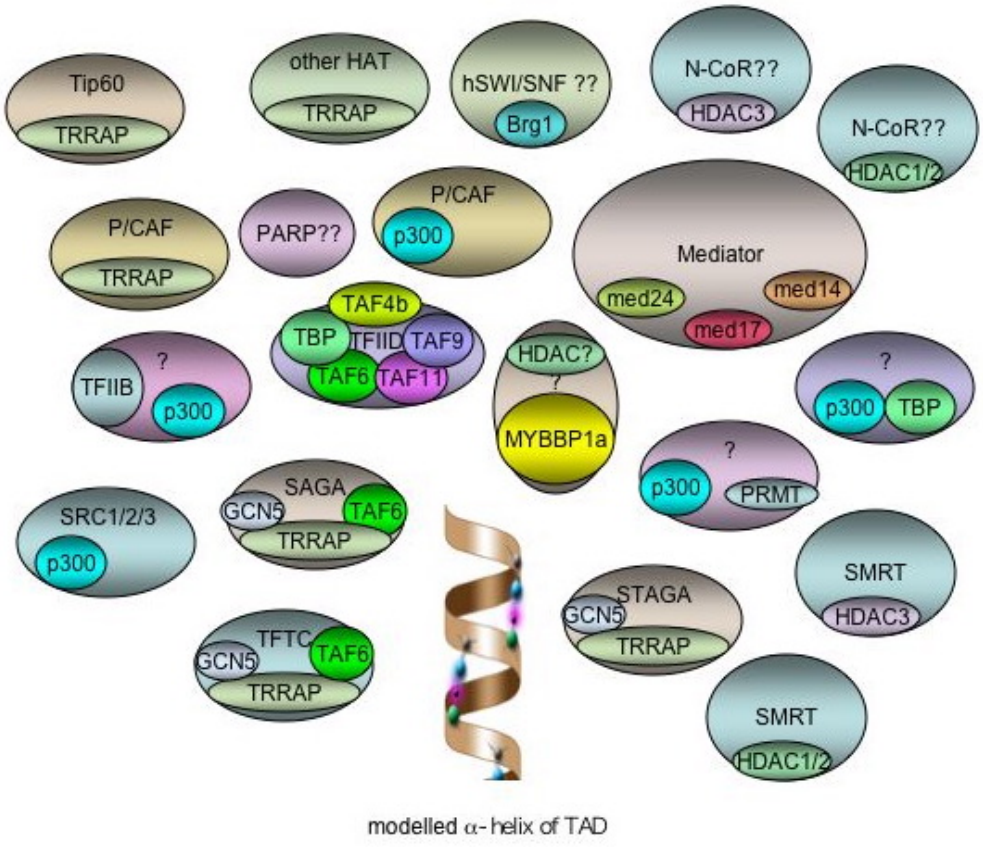


Figure 16. Protein complexes with the ability to interact with RelA(p65)-TAD. Proteins known to interact with RelA(p65)-TAD are **TRRAP** (present in TFTC, SAGA, STAGA, tip60, P/CAF and others HAT complexes), **p300** (present in SRC, PRMT, TBP, TFIIB, P/CAF HAT complexes), **MYBBP1a** (possibly in HDAC containing complexes), **TAFs** and **TFIIB** (present in TFIID, plus some other HAT complexes), **SMRT** (present in HDAC complexes) and the **Mediator**. ? indicates that the components of the complex are unknown. Other complexes involved in RelA(p65) transcription are NCoR, HDAC 1/2/3 containing complexes, hSWI/SNF complexes and PARP complexes. These complexes have not been identified to interact with RelA(p65)-TAD, and are labelled with ??. Complexes are shown as ovals and are not to scale. Members of the complex known to interact with RelA(p65)-TAD and catalytic subunits are named.

Since many proteins were found to bind to the TAD of RelA(p65), an important aspect to consider is whether only *one* protein can interact with the TAD at once, or whether *many* proteins can interact with the TAD. In a model where only one protein can interact with the TAD at once, many indirect interactions could result in the formation of a higher order complex. Many RelA(p65) proteins and other TFs are present in a single enhanceosome, which means even with only one protein binding at a time to the TAD, effective complex recruitment could occur. This is modelled for multiple Mediator subunit/TF interactions in Figure 17A. However it is unlikely that only one protein can bind the TAD at once. There are 3.6 residues per turn of a α-helix, each residue exposing its side group to the solvent. Each side chain has the possibility to form bonds with side chains from other proteins, and there is enough surface space for more than one protein to interact. Also, the TAD has two potential α-helices (one in TA1 and one in TA2) separated by 50 amino acids of presumably random coil [111, 112]. Therefore, it is likely that the TAD can interact with more than one protein at once, and this is shown in the model in Figure 17B.

Weak interactions of different subunits in the same complex with RelA(p65)-TAD would lead to a strong interaction with the whole complex. This could explain the weak interaction detected between RelA(p65)-TAD and Med14, Med17 and Med24 of the Mediator complex (Section 5.1), and also RelA(p65)-TAD interaction with multiple components of the TFIID complex (TBP, TAF6, TAF1, TAF11). It would be possible that building up of a transcription complex at an enhanceosome could involve the shift from a basally active complex to an induced transcriptionally active one, depending on the type and number of transcriptional co-activator complexes present.

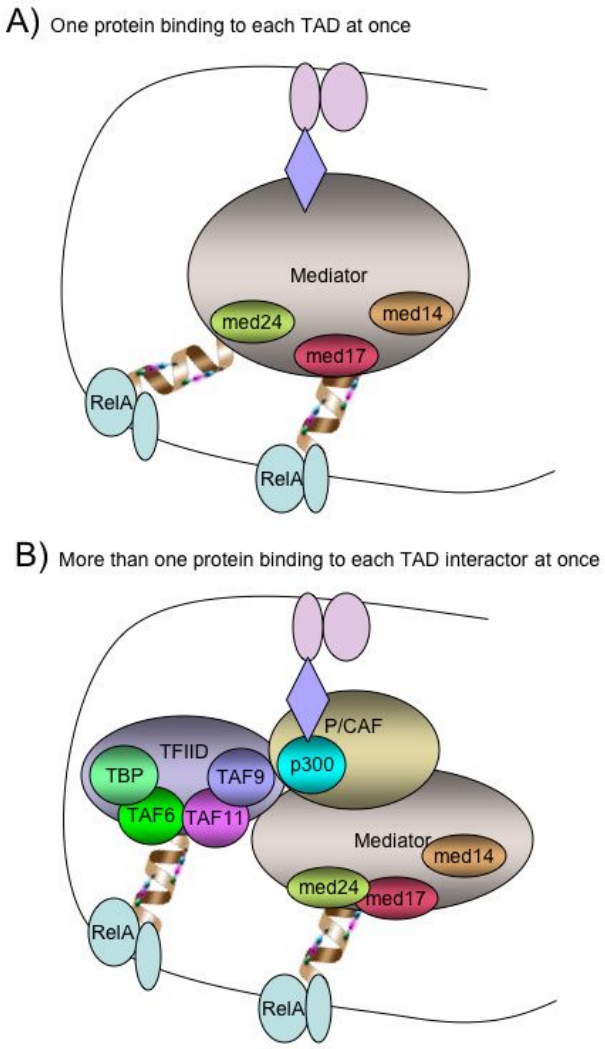


Figure 17. Model of enhanceosome function when each TAD can interact only with one protein at a time (A) or with more than one protein at a time (B). In (A) one TAD contacts Med17 and another TAD contacts Med24. Another transcription activation domain from a different TF (diamond) also contacts Mediator. In (B) one TAD can interact with Med24 and Med17, while another TAD can interact with TAF6 and TAF11. Another TF can interact with a p300 complex.

Different transcriptional cofactor complexes are used in the same enhanceosome, normally by a series of ordered recruitment events. It has been proposed that the order of cofactor recruitment to NHRs may be hSWI/SNF, followed by HATs, and then GTFs [72]. The evidence for this comes from multiple *in vitro* transcription experiments. For example, RAR/RXR dependent transcription from purified chromatin templates was synergistically activated by p300 and hSWI/SNF [292]. Order of addition experiments revealed that transcription was optimal when RXR/RAR, then hSWI/SNF, then p300 and SRC2 and finally nuclear extracts (containing GTFs) were added in order [292]. In a different system, transcription of

chromatinised reporter genes activated by the thyroid receptor- βA in Xenopus oocytes required first nucleosome remodelling, then p300 action [293]. Also ACF (ATP-utilizing chromatin assembly and remodeling factor) remodelling preceded p300 mediated acetylation during transcription activated by the acidic transcriptional activator Gal4-VP16 [294]. For NF-κB and Sp1 dependent transcription *in vitro* of the interferon regulatory factor 1 promoter, p300 HAT activity and acetyl CoA were found to be required for transcription initiation indicating p300 must be recruited to the promoter before active PIC formation [165, 295].

In vivo, ChIP experiments are used to determine the order of cofactor recruitment to specific promoter and enhancer regions, along with the pattern of histone modifications. For NF-κB regulated genes the order of cofactor recruitment to different endogenous gene control regions is not always the same, and does not strictly follow the TF, hSWI/SNF, HAT, GTF order. For example, upon virus infection NF-κB and other TFs were found to bind the INF-β enhancer after 2 hours, recruit hGCN5 HAT complexes, then CBP/RNAPII complexes, then hSWI/SNF complexes and finally GTFs to initiate transcription after 6 hours [296]. In contrast, hSWI/SNF was already present before TNFα stimulation at the E-selectin promoter [210]. After 30 min, hSWI/SNF levels decreased whilst NF-κB, IKKα and HDAC2 levels were increased. TBP was present at the transcriptional start site whilst p300 and SRC1 were present downstream of the start site. At 1 hour after treatment, most proteins were not detected apart from P/CAF and RNAPII [210]. These studies support the model that cofactor requirement and the order of complex recruitment to NF-κB regulated genes may be dependent on the promoter context.

The ChIP studies of NF-κB regulated genes often compare cofactor presence at time points varying by around 30 minutes. This could result in a simplified view of cofactor recruitment, as studies with other TFs have shown a more complex cycle of cofactor recruitment events. A ChIP study in five minute intervals of cofactor recruitment to the estrogen receptor (ER) responsive pS2 promoter showed that there were three cycles of events, leading to effective transcription [80]. The first cycle was found to be unproductive and involved recruitment of ligand bound ER, followed by hSWI/SNF, PRMT, HATs and GTFs. This cycle ended in the degradation of ER, but upon re-binding of ER, the following cycles were found to be transcriptionally productive. After recruitment of GTFs, RNAPII closely followed by Mediator is

associated with the promoter region. There is evidence that transcription regulated by NF-κB also occurs in cycles on many different promoters [119, 181, 297]. When considering the mechanism of NF-κB cycles it should be kept in mind that most nuclear NF-κB binds to DNA dynamically, interacting with the DNA for just a few seconds as measured by fluorescence loss in photobleaching experiments [297]. This indicates that any enhanceosome structure component could be in constant flux with the surroundings, but that the equilibrium is towards the well characterised cofactor complexes detected when analysing a single time point by ChIP experiments.

Together, these studies indicate that there are a vast number of transcriptional cofactor complexes for RelA(p65). The cofactor requirement and the cyclic order of recruitment of different RelA(p65) cofactor complexes to enhancers is likely to dependent on many factors. These factors could be the chromatin structure surrounding the gene and its regulatory elements, the other TFs present, the cell type, the stimuli and position in the cell cycle.

10.2 TAD cofactor exchange by PTMs

Exchange of co-repressor complexes for co-activators, or co-activators for other co-activators occurs during transcription activation. Cofactor complex exchange could be through signal specific post translational modifications (PTMs) of TFs allowing regulated interactions with cofactors. For example, PKA mediated phosphorylation of RelA(p65) at serine 276 increased p300 interaction, but reduced HDAC1 interaction [298]. The TAD of RelA(p65) has many serines that have been shown to be phosphorylated upon cell stimulation, and result in the increase of the transactivation potential of RelA(p65) (Section 2.5.1). Calcium/Calmodulin-dependent protein kinase IV (CaMKIV) mediated phosphorylation of GST-RelA(p65)-TAD led to an increase in p300 binding and a decrease in co-repressor SMRT binding [131]. The phosphorylated serine was mapped to be serine 535 *in vitro*, and mutation of this residue to alanine prevented phosphorylation by CaMKIV, p300 binding and SMRT release [132]. In a different study CHIP experiments showed that laminin stimulation of cells led to IKKα dependent phosphorylation of SMRT and serine 536 of RelA(p65) at the cIAP-2 promoter [119]. The phosphorylation events correlated with a decrease in HDAC3 association with the promoter region, and increase in RNAPII association. Following RelA(p65) phosphorylation, and HDAC3 release, acetylation

of RelA(p65) at serine 310 occurred, which was shown to be mediated by p300. Therefore, the specific phosphorylation of RelA(p65) and SMRT co-repressor, led to HDAC release and HAT activity recruitment [119]. TNFα was also found to stimulate p300 interaction with wild-type RelA(p65), but not with non-phosphorylatable mutants S276A and S536A, as detected by co-immunoprecipitation [299]. More experiments are required to determine whether other cofactor interactions with RelA(p65)-TAD are also affected by site specific, signal induced PTMs.

Cofactor exchange may also require more than a change in PTMs of RelA(p65). TBL1 and TBLR1 were found to be required for NF-κB dependent transcription [300]. These proteins were identified as co-repressor/co-activator exchange factors that are recruited to promoters upon transcription induction and cause the exchange of cofactors through ubiquitin mediated degradation of factors [300].

10.3 Regulation of MYBBP1a interaction with RelA(p65)

MYBBP1a interacts with RelA(p65) to repress NF-κB regulated transcription, while p300 interacts with RelA(p65) to activate transcription. Further experiments are required to determine whether the interaction between RelA(p65) and MYBBP1a or p300 is regulated to control the expression of specific genes. It would be expected that the levels of MYBBP1a available in the nucleus to interact with RelA(p65) could be one way to regulate the transcriptional outcome of RelA(p65). MYBBP1a is expressed differentially between cell types, and is higher in Jurkat T cells than in HeLa epithelial or THP1 monocyte cells, indicating that a larger pool of RelA(p65) may be associated with MYBBP1a in Jurkat T cells. This could lead to repression of certain NF-κB genes in these cells. The cellular localisation of MYBBP1a would also be expected to regulate the MYBBP1a/RelA(p65) interaction. MYBBP1a was shown to shuttle between the nuclear/nucleoli and cytoplasm [235]. Preliminary experiments showed that PMA stimulation of Jurkat cells led to a pool of MYBBP1a relocating from the nucleolus to the cytoplasm and nucleoplasm (Section 6.1). PMA activates members of the protein kinase C (PKC) family and other effectors. These effectors signal via many different kinases such as IKK and p38 MAPK (mitogen activated protein kinase) to cause a wide variety of cellular responses including cell death, differentiation, transformation, proliferation, inflammation, apoptosis, adhesion,

survival and motility depending on the cell type and context (reviewed [301, 302]). Extension of these preliminary MYBBP1a localisation experiments is required to investigate the mode of MYBBP1 mobilisation in the nucleus and its effect on NF-κB dependent transcription.

Another level of regulation of the MYBBP1a/RelA(p65) interaction versus the p300/RelA(p65) interaction could be through regulation of PTMs of RelA(p65), MYBBP1a or p300. This would be similar to the regulation of the MYBBP1a interaction with PGC-1α, which affects the potential of PGC-1α to activate transcription. PGC-1α is phosphorylated by p38 MAPK at serine residues in its negative control region upon p38 MAPK induction by cytokines and β-adrenergic signals [303]. Phosphorylation of PGC-1α by p38 MAPK *in vitro* reduced MYBBP1a binding [274]. *In vivo*, TNFα, IL-1α and IL-1β stimulation reduced MYBBP1a co-immunoprecipitating with PGC-1α and this coincided with p38 MAPK phosphorylation of the specific sites in PGC-1α. MAP kinase kinase 6, an upstream activator of p38 MAPK, relieved repression of MYBBP1a [274]. Also, the mutant of PGC-1α that mimics phosphorylated, activated PGC-1α was found to be resistant to MYBBP1a repression. Interestingly IL-1β stimulation leads to p38 MAPK activation and transcription activation by RelA(p65) dependent on the phosphorylatable serines 529 and 536 of RelA(p65) [122]. p300 and Akt (the MAPK activator) synergistically enhance reporter transcription [122]. This leads to the model that some signals such as IL-1β could activate phosphorylation of RelA(p65) at serine 536, resulting in an increase of p300 interaction with TAD. This might also coincide with a decrease in MYBBP1a binding, since MYBBP1a was shown to compete with p300 for interaction with the TAD (Section 5.2). Alternatively other signal specific inducible PTMs of RelA(p65) could regulate the exchange of p300 and MYBBP1a.

A model is proposed in Figure 18, suggesting a mechanism for cell type specific regulation of certain genes by MYBBP1a/p300 exchange. In cells with high levels of MYBBP1a (e.g. Jurkat T cells), activation of NF-κB nuclear import (by stimulus X in Figure 18), may lead to activation of some RelA(p65) regulated genes (such as IκBα) but not others that are repressed by MYBBP1a (gene A in Figure 18A). MYBBP1a may repress the specific genes by interacting with TAD only at the enhancers of specific genes. Alternatively MYBBP1a containing HDAC complexes may only be repressive in certain promoter environments. Stimulation of T cells by another signal,

such as IL-1β (stimuli Y in Figure 18), could lead to phosphorylation of the TAD at specific residues (probably serine 536 for IL-1β). This phosphorylation would increase the p300/TAD interaction, perhaps with a decrease in MYBBP1a/TAD interaction allowing transcription activation at these specific genes (Figure 18B). In another cell type, where low levels of MYBBP1a are expressed (e.g. epithelial cells such as HeLa), stimulation of NF-κB nuclear import with factor X could activate both types of gene, since MYBBP1a is not available to repress transcription (Figure 18C). Upon stimulation with the second signal Y (e.g. IL-1β) the gene could be fully activated by phosphorylation of the TAD enhancing p300 recruitment (Figure 18D). This model shows how specific RelA(p65) regulated genes could be differentially regulated according to cell type due to a transcriptional repressor whose expression is restricted to certain cell types.

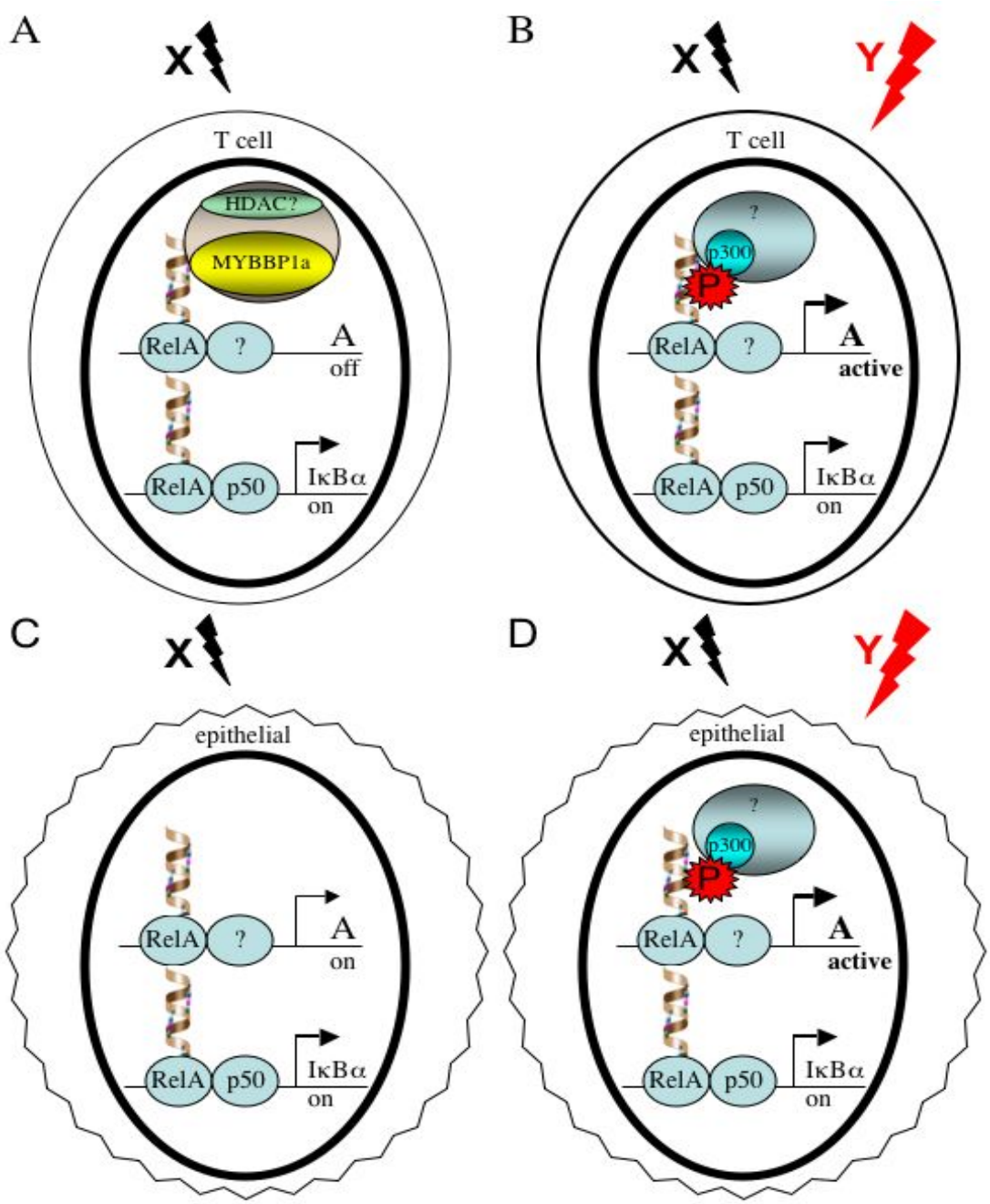


Figure 18. Model to illustrate that MYBBP1a could repress subsets of genes in specific cell types. (A) In T cells, MYBBP1a expression is likely to be high. Upon T cell treatment with factor **X** (black flash) certain RelA(p65) genes could be activated such as IκBα. Others genes (shown as gene A) may be repressed by MYBBP1a interaction with the TAD (shown as a helix). MYBBP1a may be associated with HDAC complexes and can compete with p300 binding to the TAD. (B) In T cells, treatment with **X** and **Y** (e.g. IL-1β, red flash) may lead to phosphorylation of serine 536 and p300 interaction with the TAD of RelA(p65). It is possible that the MYBBP1a/TAD interaction could also be reduced, enabling transcription activation of gene A. (C) In cells with less MYBBP1a (possibly epithelial), MYBBP1a is not available to repress gene A, which could then be transcribed upon treatment of the cell with only factor **X**. (D) Treatment of cells with low MYBBP1a levels with factor **Y** (e.g. IL-1β) could lead to phosphorylation of TAD and p300 interaction, and further activation of gene A.

10.4 Perspectives

The results presented in this thesis, together with previous studies identify MYBBP1a as a transcriptional repressor differentially expressed in certain cell types. In particular high levels of MYBBP1a were found in Jurkat T cells. Currently, it is not known under which conditions MYBBP1a represses endogenous gene expression, or whether MYBBP1a represses the majority or only a subset of RelA(p65) regulated genes. This could be investigated by micro-array analysis of mRNA expressed under different conditions in siRNA mediated MYBBP1a knock down cells. This knowledge, together with identification of the expression pattern of MYBBP1a normal tissues would help us to understand the contribution of MYBBP1a to NF-κB cell type specific transcription and which cellular processes are regulated by MYBBP1a.

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